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Ciplys et al.

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(54) **GENERATION OF NATIVE RECOMBINANT SECRETED HUMAN ENDOPLASMIC RETICULUM CHAPERONES BY USING THEIR NATIVE SIGNAL SEQUENCES IN YEAST EXPRESSION SYSTEMS**

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(65) **Prior Publication Data**

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Related U.S. Application Data

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(60) Provisional application No. 61/670,768, filed on Jul. 12, 2012.

(51) **Int. Cl.**
C12N 9/90 (2006.01)
C07K 14/47 (2006.01)
C12N 15/81 (2006.01)
C12P 21/02 (2006.01)

(52) **U.S. Cl.**
CPC **C12N 9/90** (2013.01); **C07K 14/47** (2013.01); **C07K 14/4702** (2013.01); **C07K 14/4728** (2013.01); **C12N 15/81** (2013.01); **C12P 21/02** (2013.01); **C12Y 503/04001** (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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(57) **ABSTRACT**

A method using yeast as a host for production of human ER chaperone proteins, using endogenous signal peptides of intracellular human proteins that are recognized and correctly processed in the yeast cells to subsequently lead to the secretion of the human proteins. The resultant proteins possessed native amino acid sequence and were biologically active. Moreover, secretion allowed simple one-step purification of native recombinant human proteins with high yields.

10 Claims, 24 Drawing Sheets

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FIGURE 1

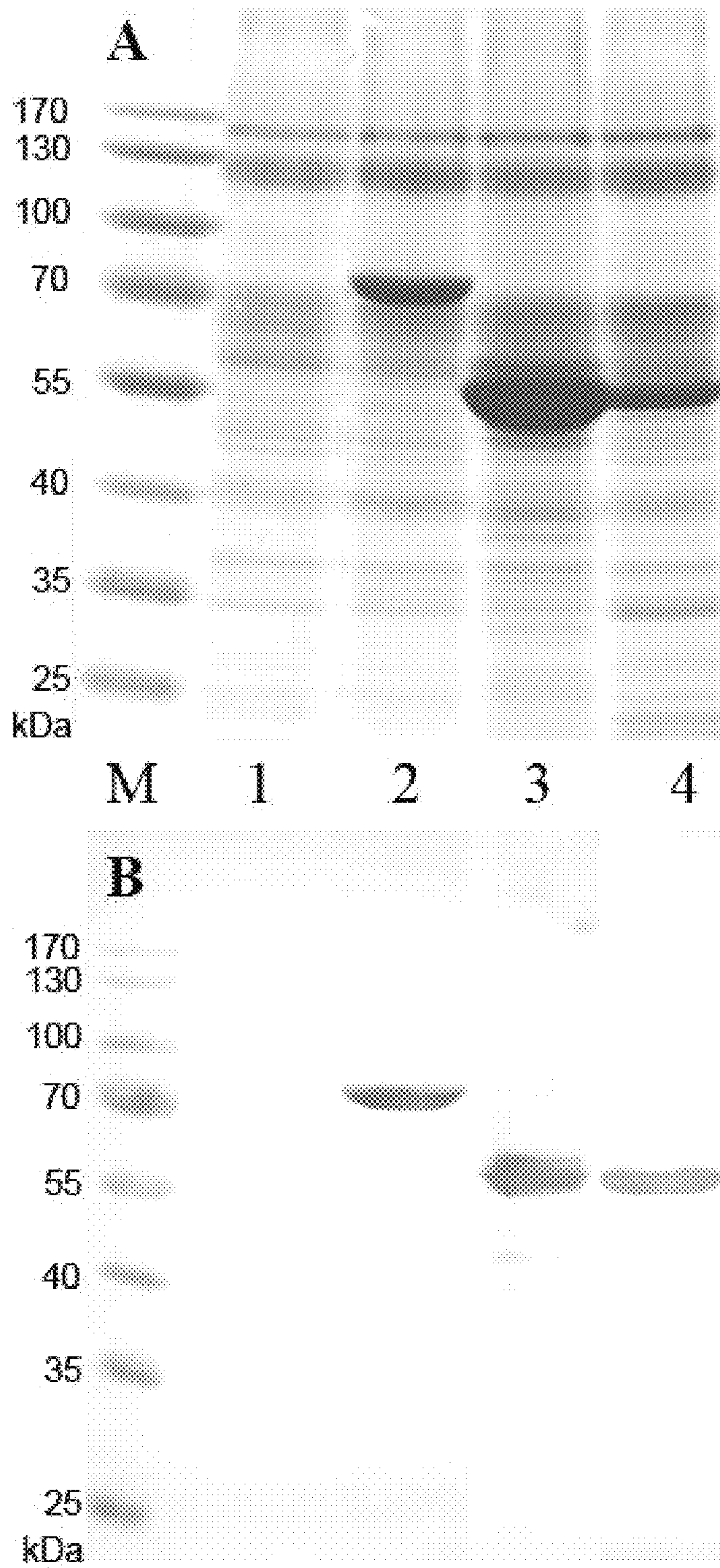


FIGURE 2A

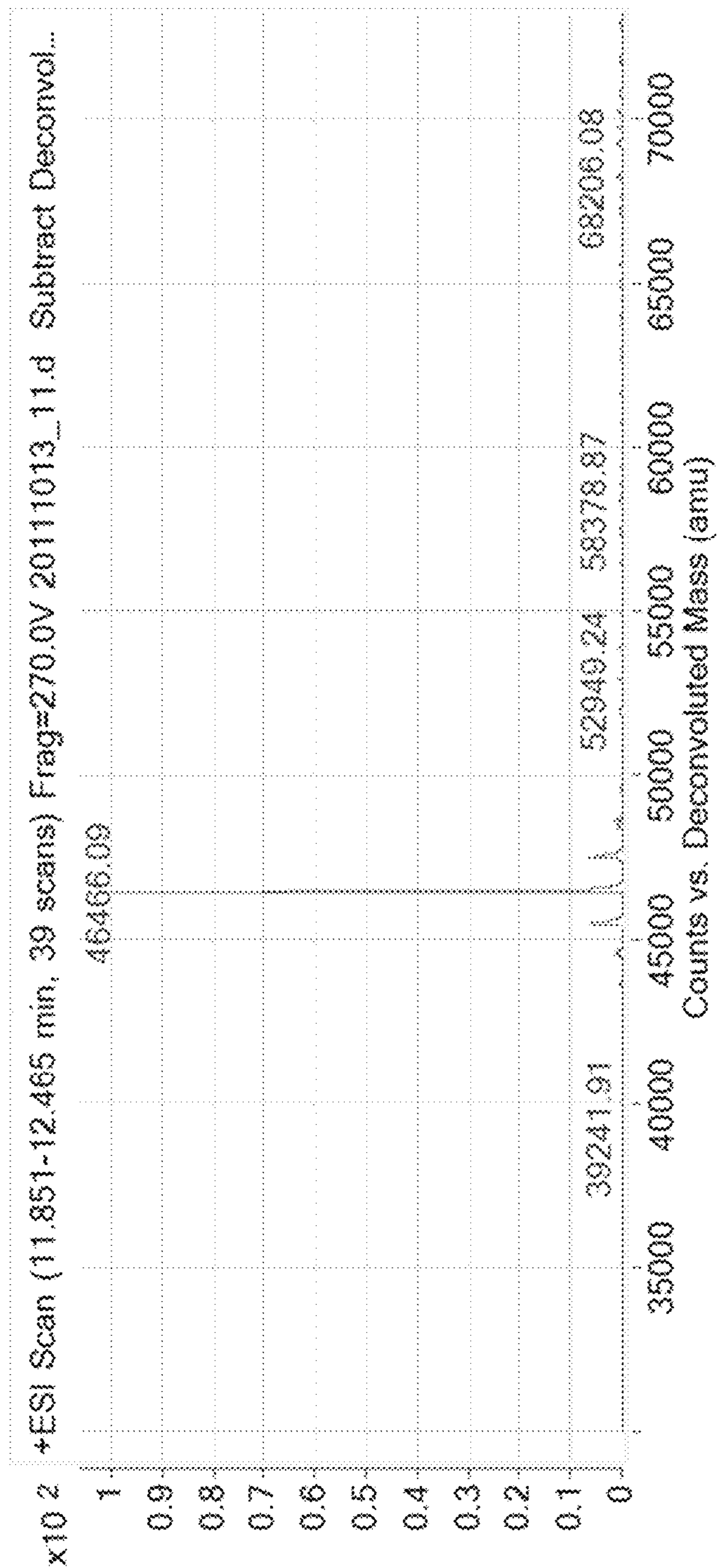


FIGURE 2B

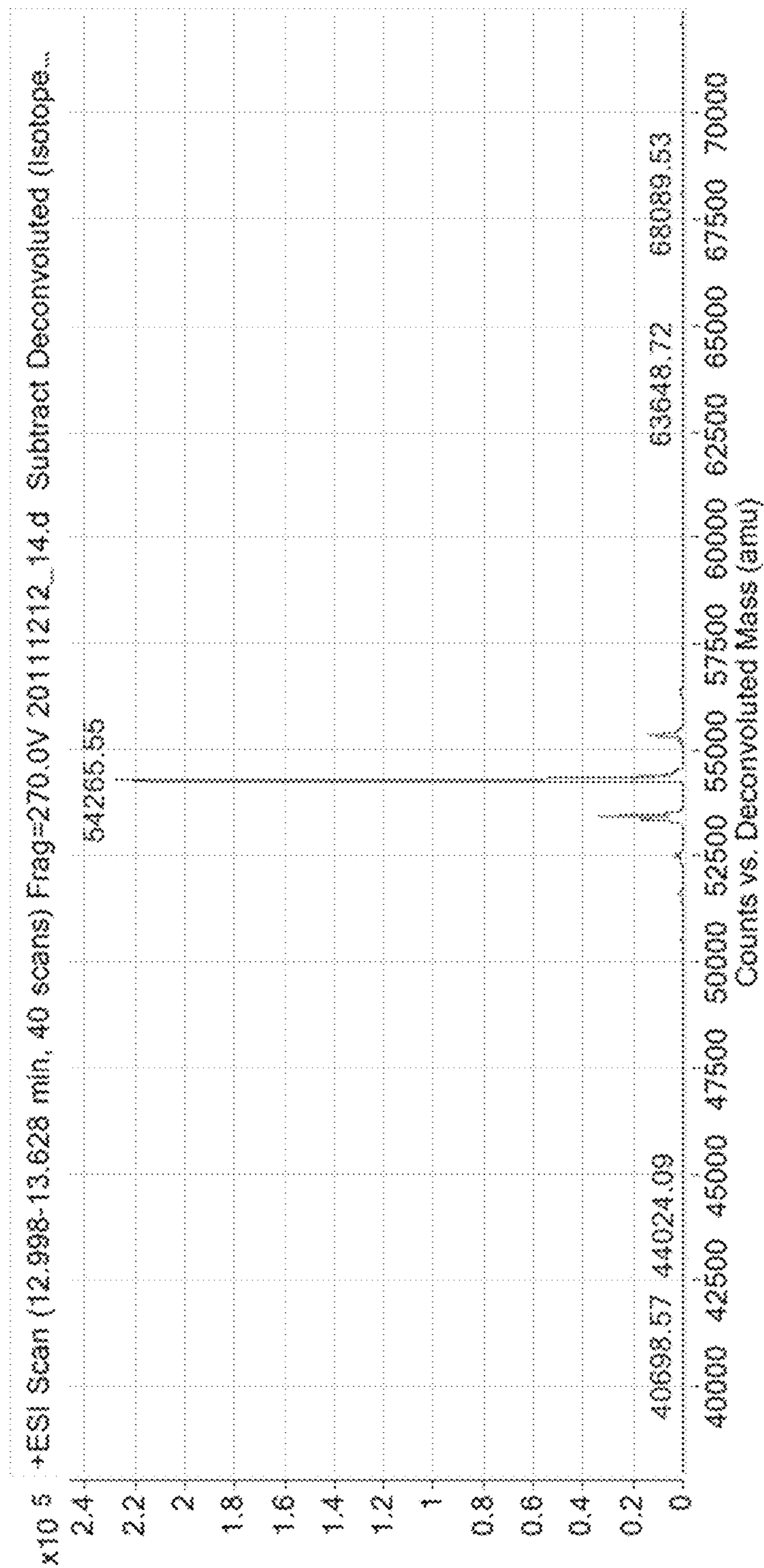


FIGURE 2C

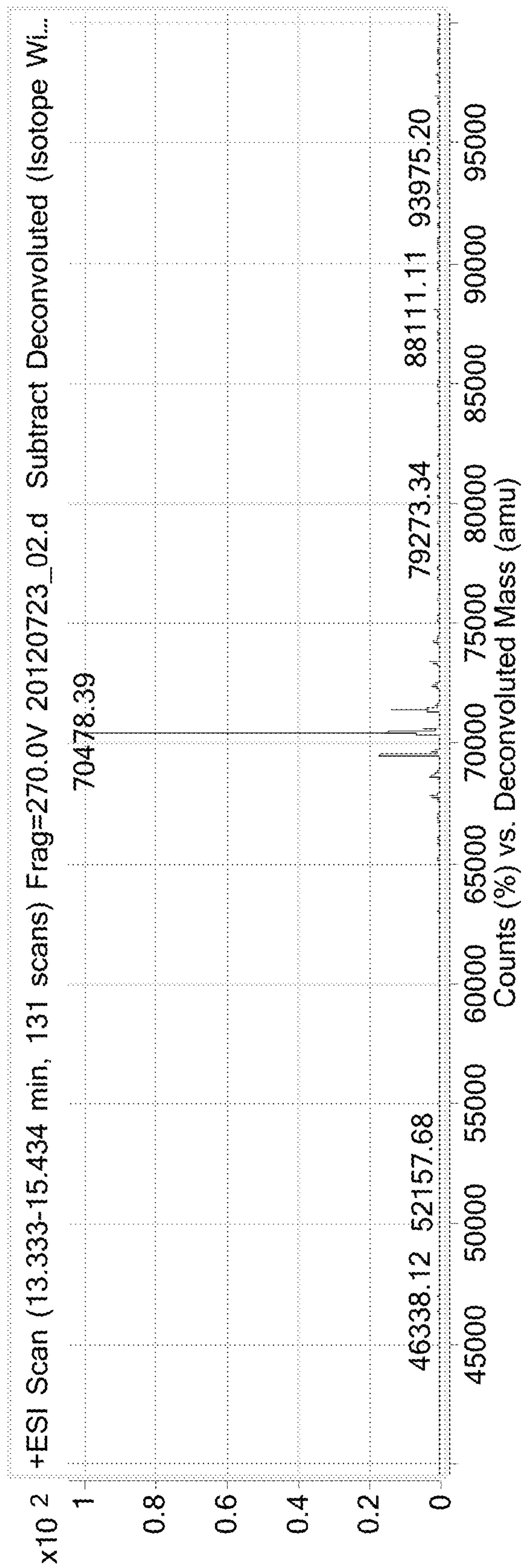


FIGURE 3

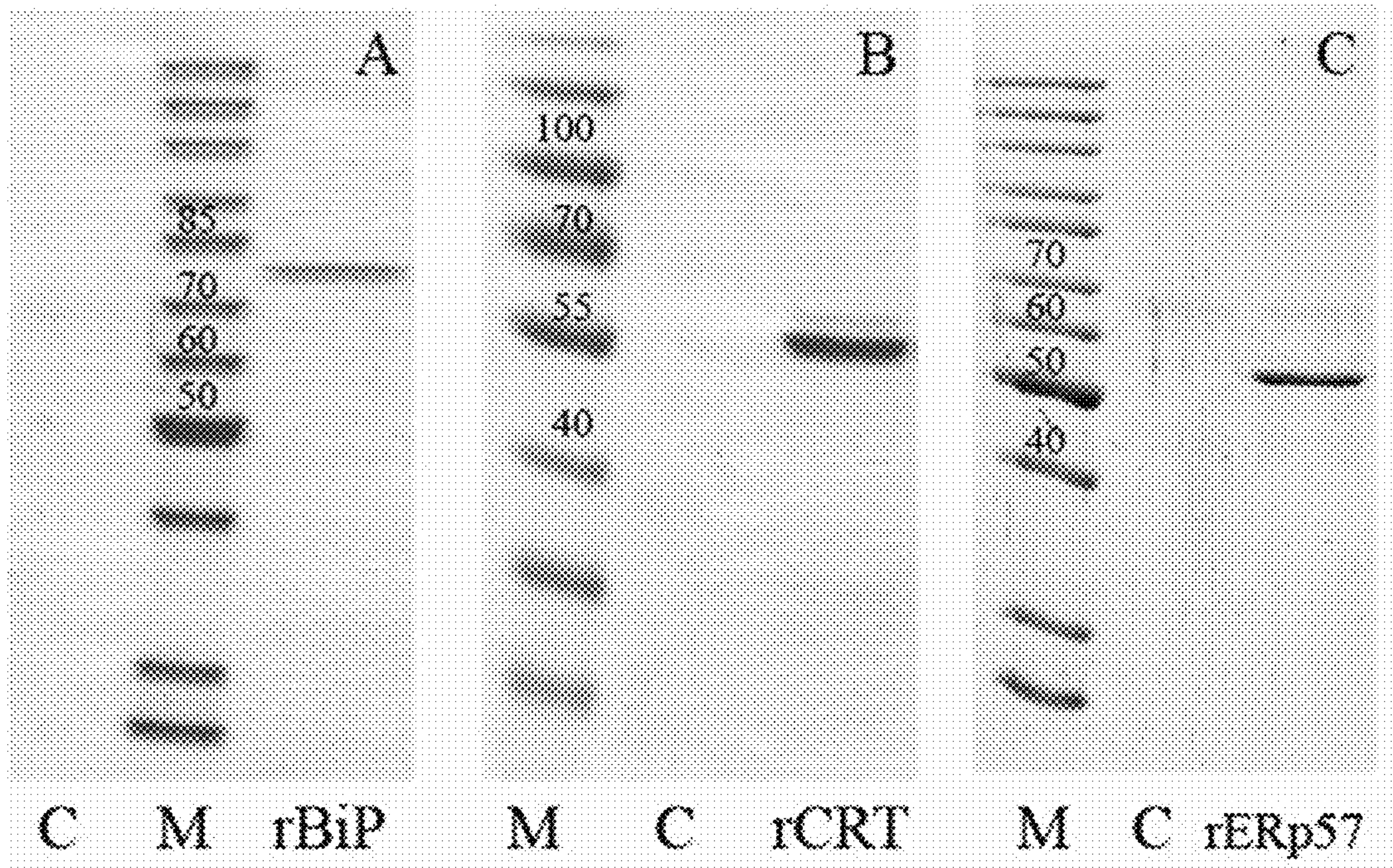


FIGURE 4

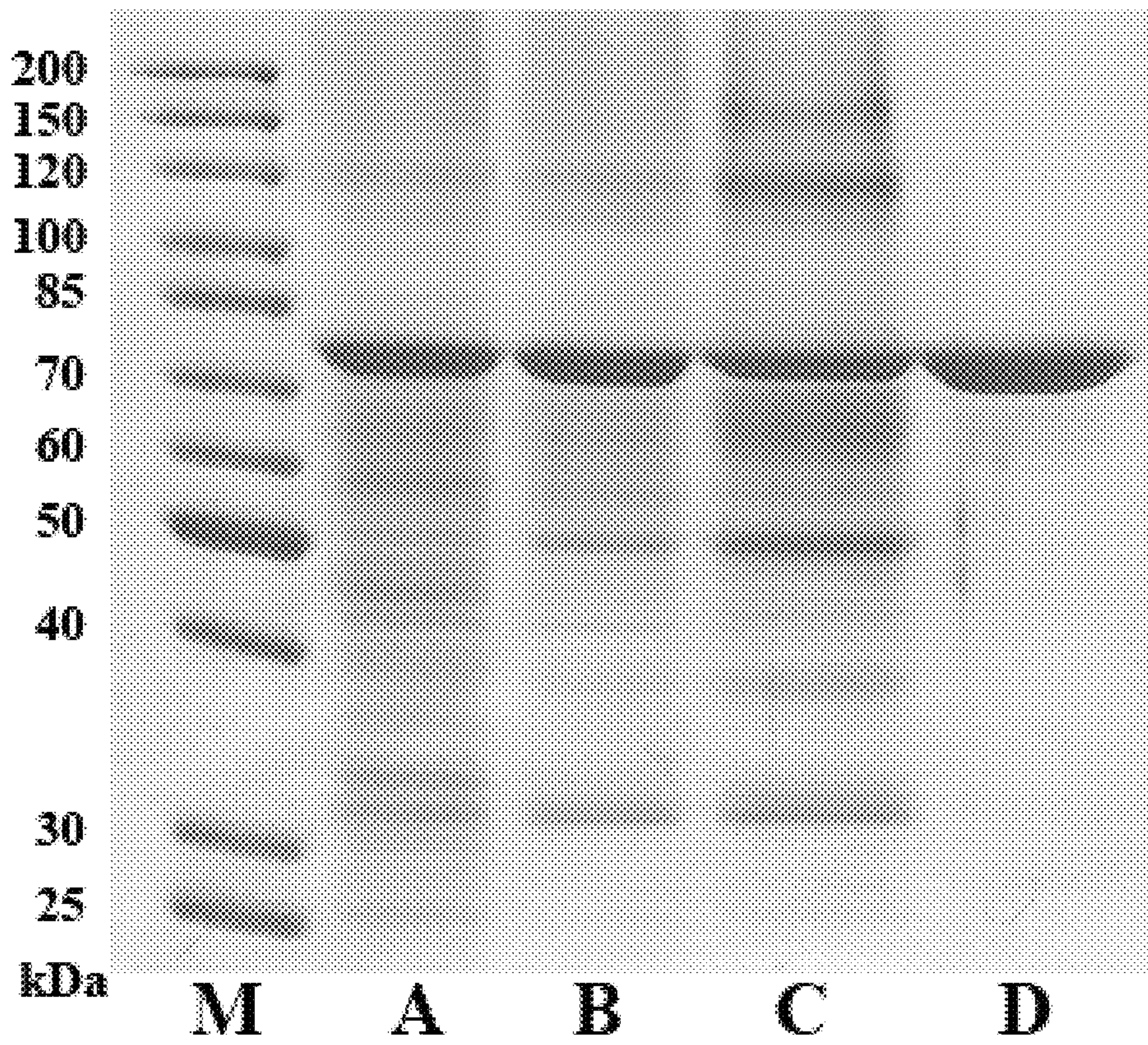


FIGURE 5

1 MKLSLVAAML LLLSAARAE EDKKEDVGTV VGIDLGTTYS CVGVFKNGRV
51 EIIANDQGNR ITPSYVAFTP EGERLIGDAA KNQLTSNPEN TVFDAKRLIG
101 RTWNDPSVQQ DIKFLPFKVV EKKTKPYIQV DIGGGQTKTF APEEISAMVL
151 TKMKETA EAY LGKKVTHAVV TVPAYFNDAQ RQATKDAGTI AGLNVMRIIN
201 EPTAAAIAYG LDKREGEKNI LVFDLGGGTF DVSLLTIDNG VFEVVATNGD
251 THLGGEDEFDQ RVMEHFIKLY KKKTKGDVRK DNRAVQKLRR EVEKAKRALS
301 SQHQARIEIE SFYEGEDFSE TLTRAKFEEL NMDLFRSTMK PVQKVLESD
351 LKKS DIDEIV LVGGSTRIPK IQQLVKEFFN GKEPSRGINP DEAVAYGAAV
401 QAGVLSGDQD TGDVLVLLDVC PLFLGIETVG GVMTKLIPRN TVVPTKKSQI
451 FSTASDNQPT VTIKVYEGER PLTKDNHLLG TFDLTGIPPA PRGVPQIEVT
501 FEIDVNGILR VTAEDKGTGN KNKITITNDQ NRLTPEEIER MVNDAEKFAE
551 EDKCLKERID TRNELESYAY SLKNQIGDKE KLGKLSSED KETMEKAVEE
601 KIEWLESHQD ADIEDFKAKK KELEEIVQPI ISKLYGSAGP PPTGEEDTAE
651 KDEL

SEQ ID NO:1

FIGURE 6

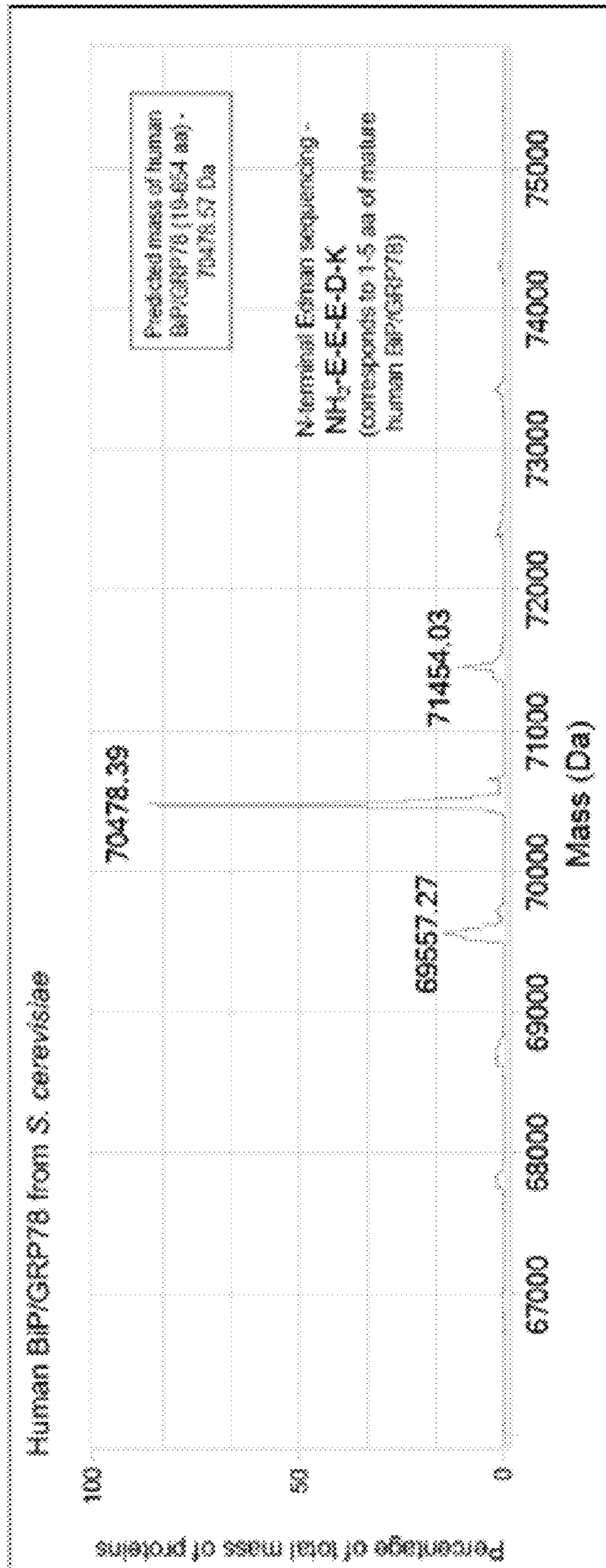


FIGURE 6 continued

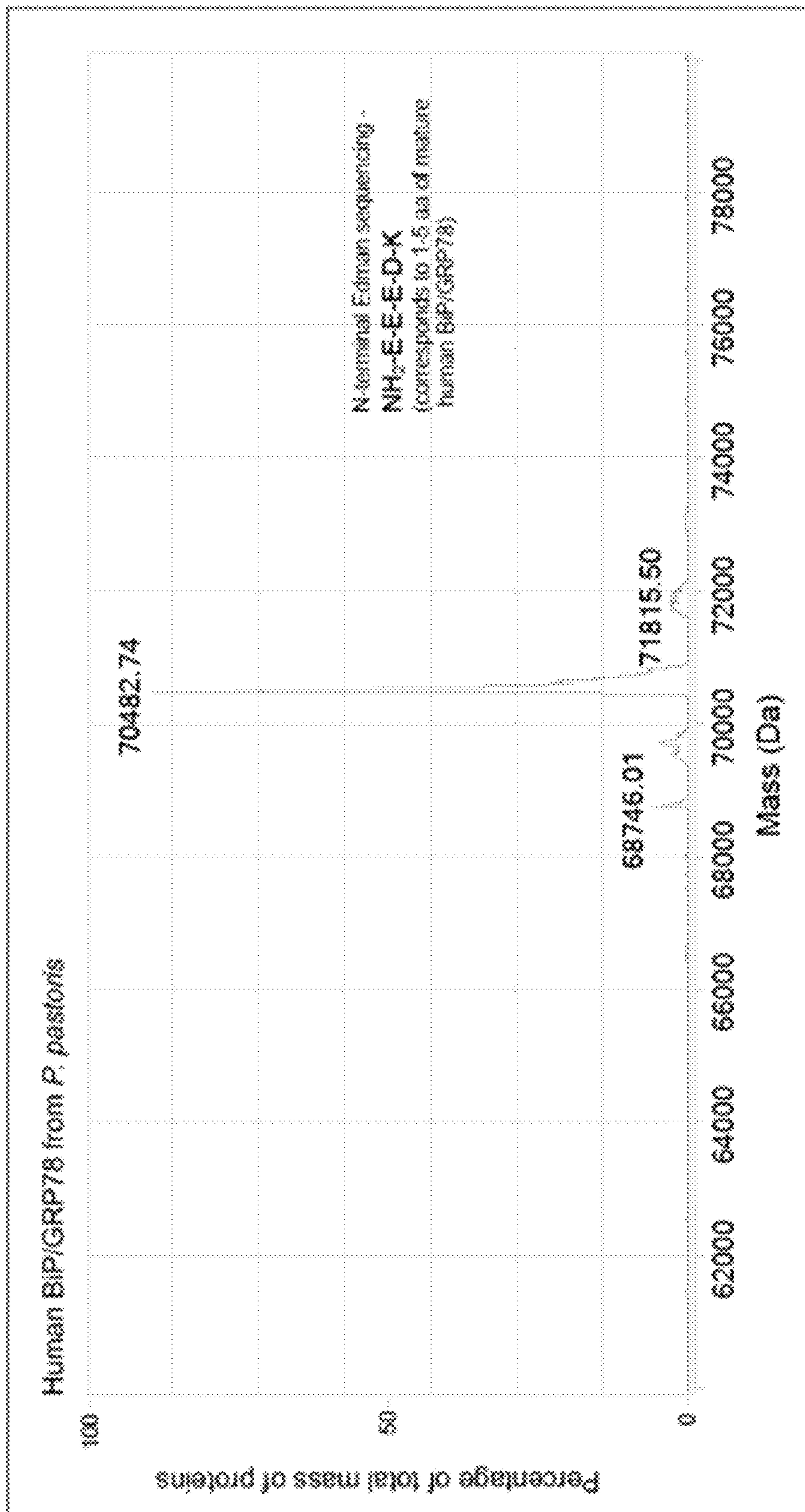


FIGURE 7

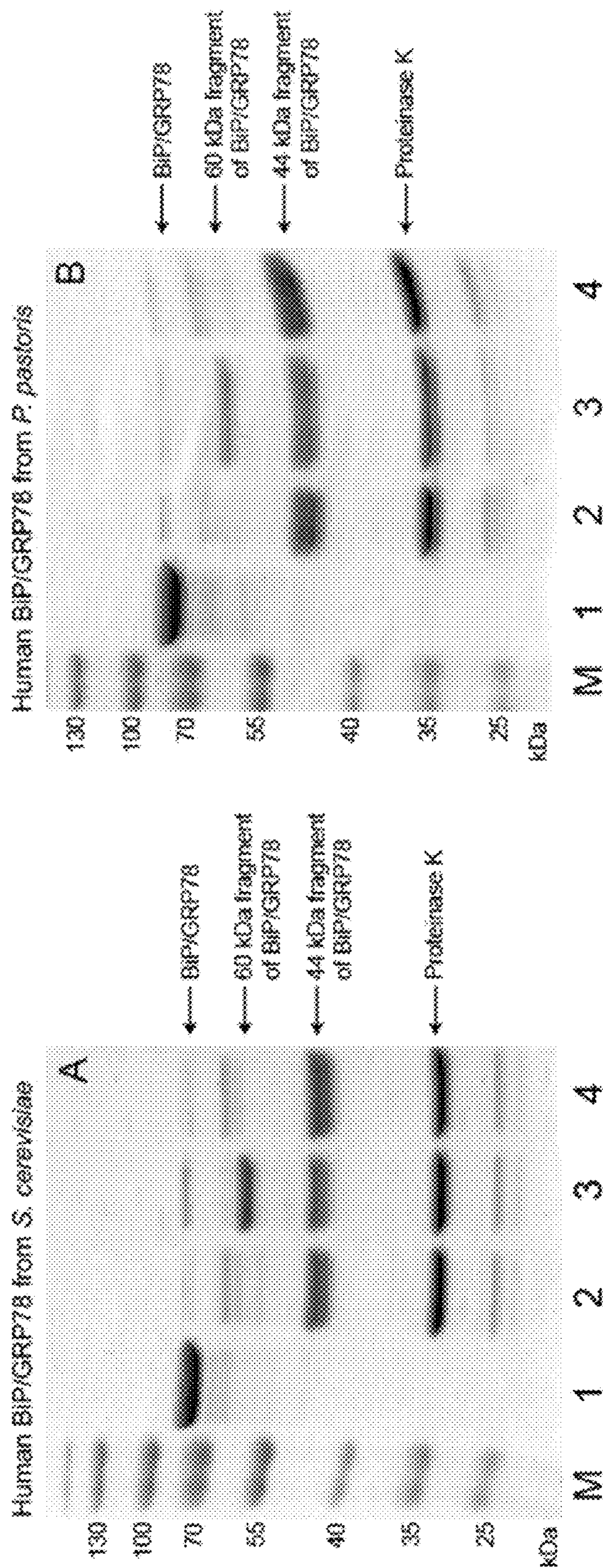


FIGURE 8

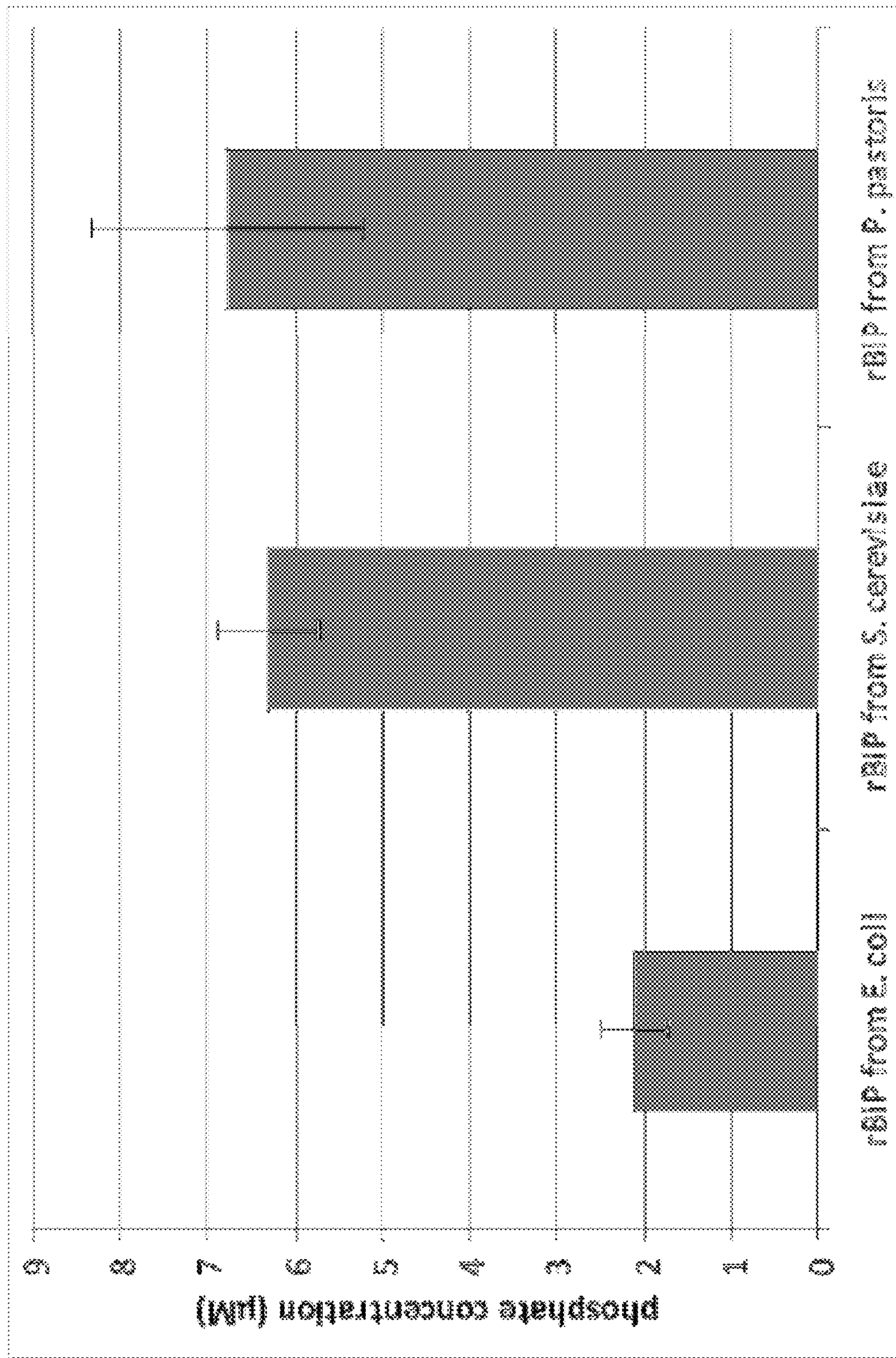


FIGURE 9

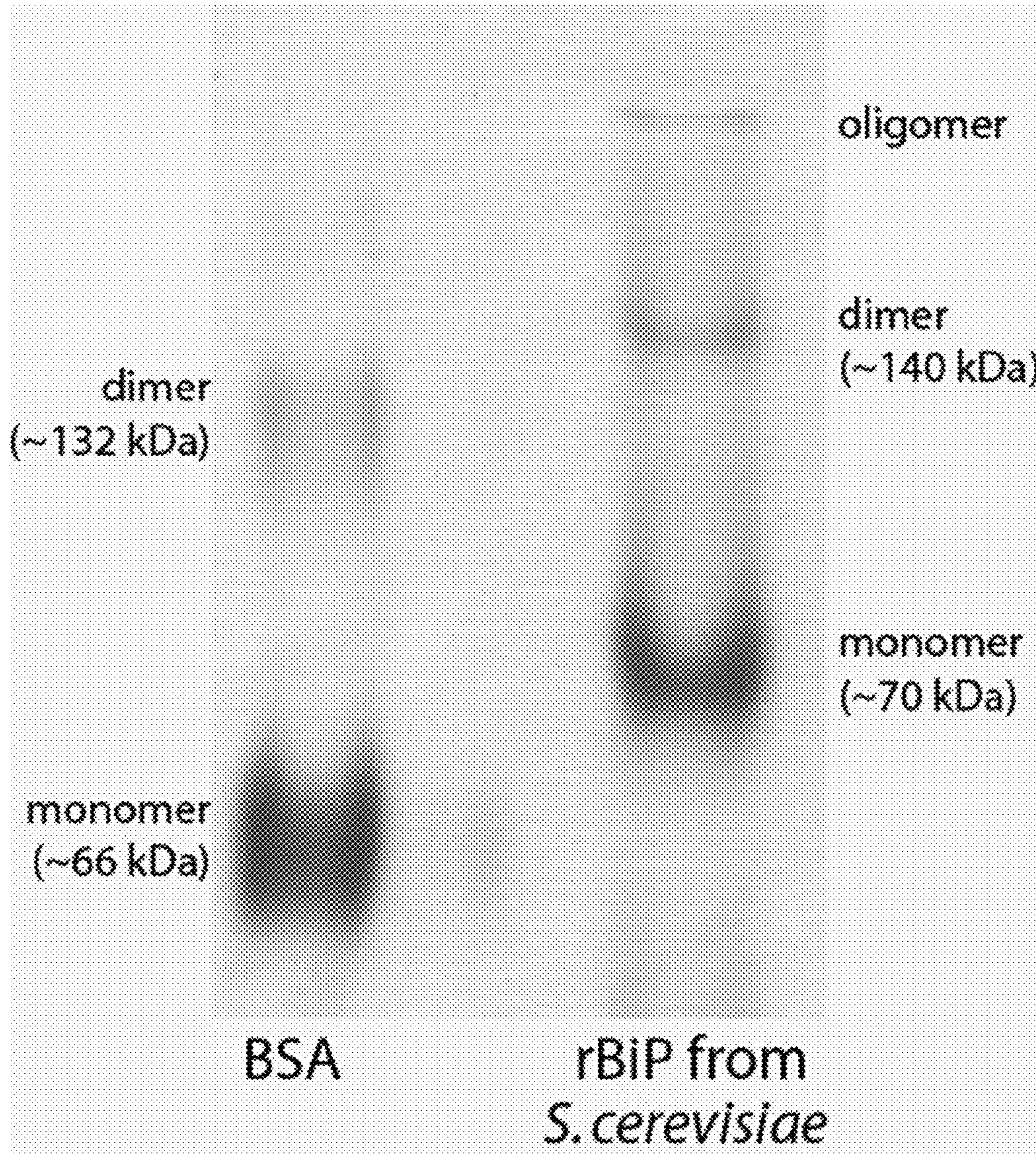


FIGURE 10

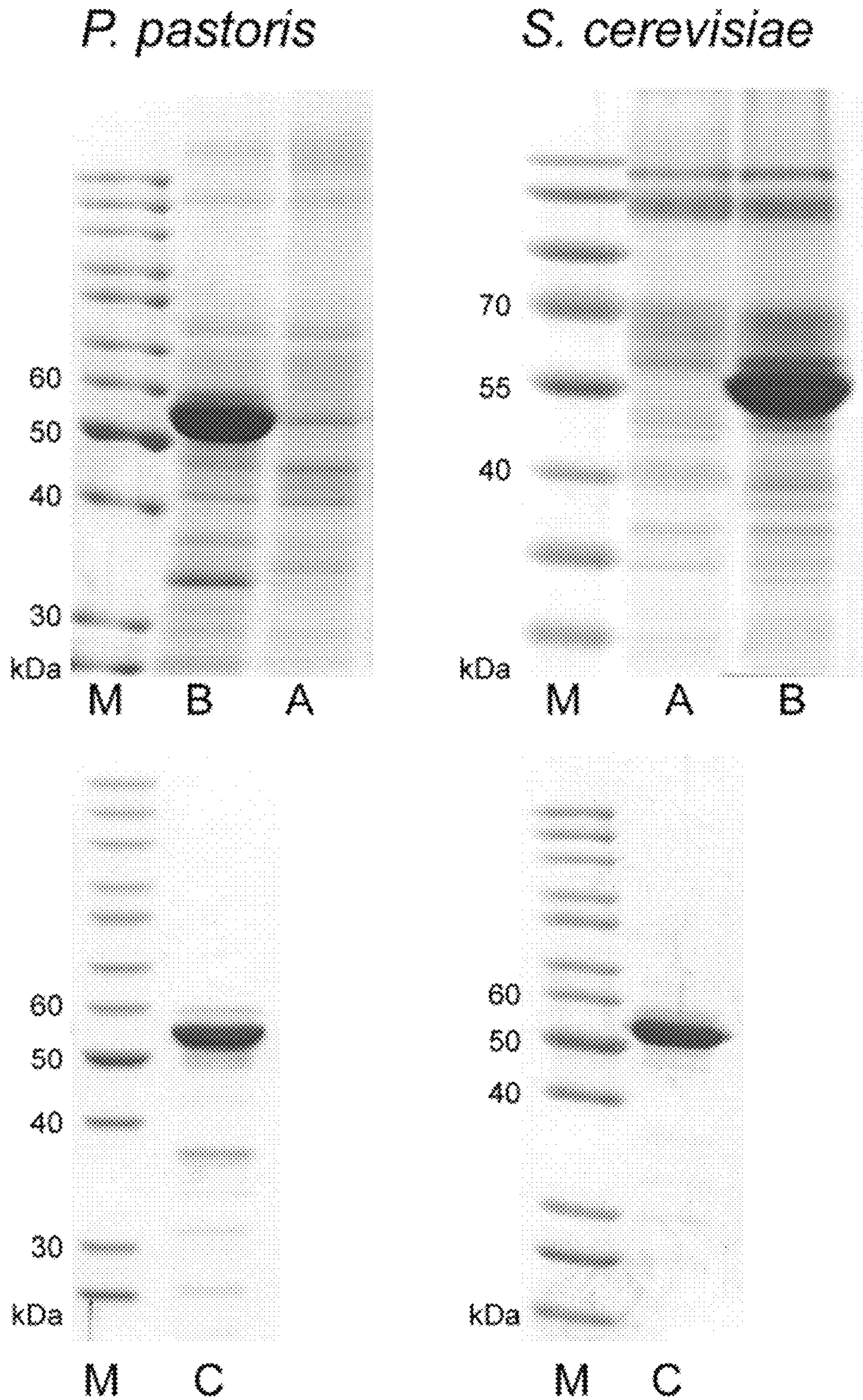


FIGURE 11

Protein CALR_Human.....P27797 Without signal peptide
 Mw 48142Da 417 aa. SEQ ID NO: 2 Mw 46466Da 400 aa.

Protein is without signal
 Sequence of 17 aa length

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1  MLLSVPDLLG LGLAVRPA VYKEQELDG DQWTSRWIES KHKSDFGKTV LSSGKFYGDDE
61  EKDKGLQTSQ DARFYALSAS FEFPSNKKQT LVVQFTVSHH QNIDCGGGYV KLFPSLDQQT
121 DMHGESEYNI MFGEDICGEG TKKVHVIENY KGRNVLINKD YRCKDDEFTH LYTLIVRPDN
181 TYEVKIDNSQ VEGGSLEDDW DELEPKKIKD PDASKPEDWD ERAKIDDFTD SKFEDWDKPE
241 HIPDFDAKKE EDWDEMDGE WEPFYQNPPE YKGEWKRQI DNPDKGTWI HPEIDNPEYS
301 PERSIYAYDN FGVIGLILWQ VKSGTIFDNF LITNDEAYAE EFGNETWGYT KAKENQMKIK
361 QDEEQRLKEK EEDKRRKEE EAEKDEDED KNEDEEDED KEEDEEEDVE GOAKDEL
  
```

Start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss Sequence
18 - 24	427.2400	852.4654	852.4381	0.0273	0 A...E...V...K...E (Ions score 38)
18 - 24	427.2700	852.5254	852.4381	0.0879	0 A...E...V...K...E (Ions score 29)

The peptide begins not from lysine or arginine.
 This indicates that it is N-terminal residue.

FIGURE 12

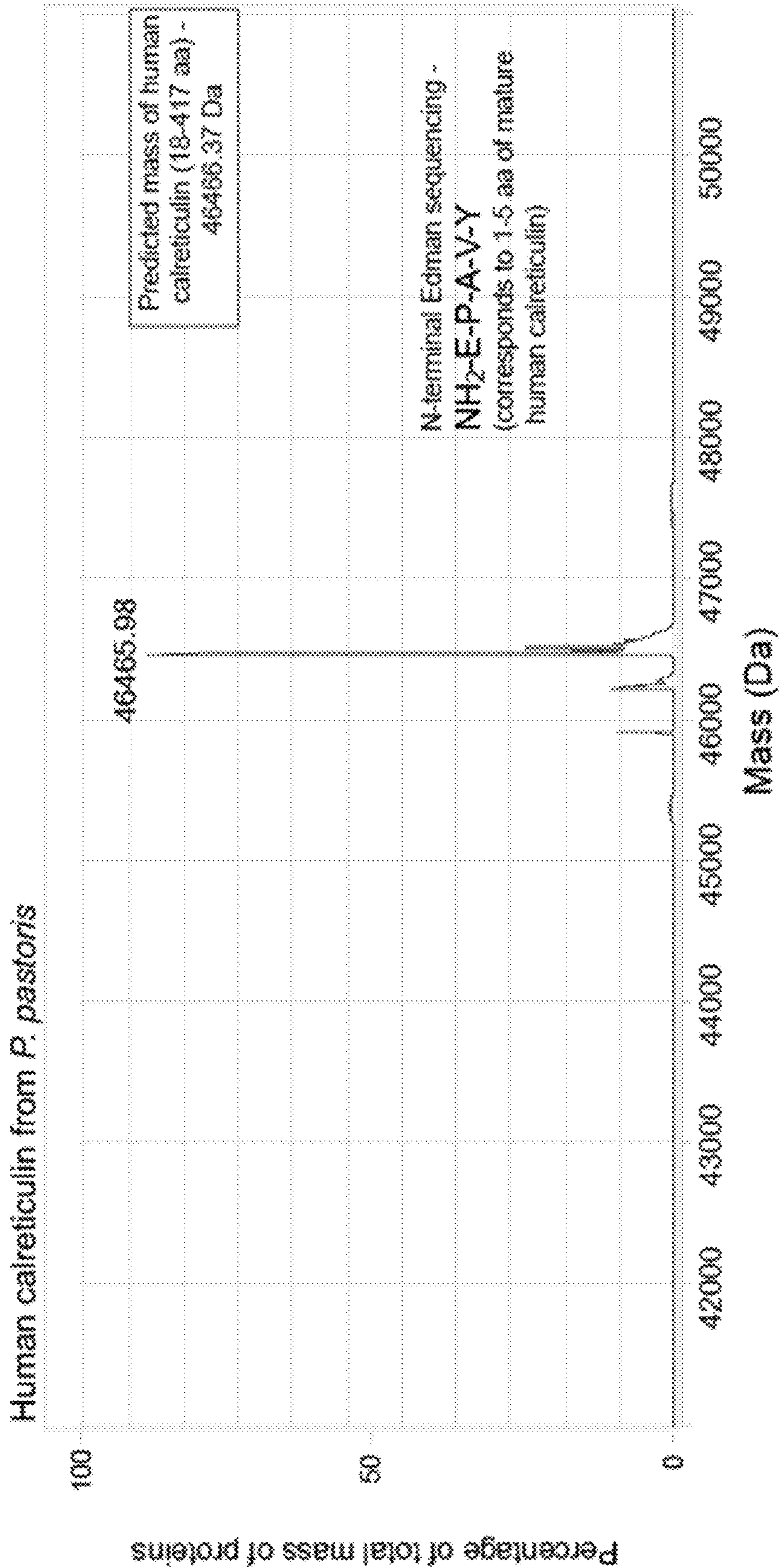


FIGURE 12 continued

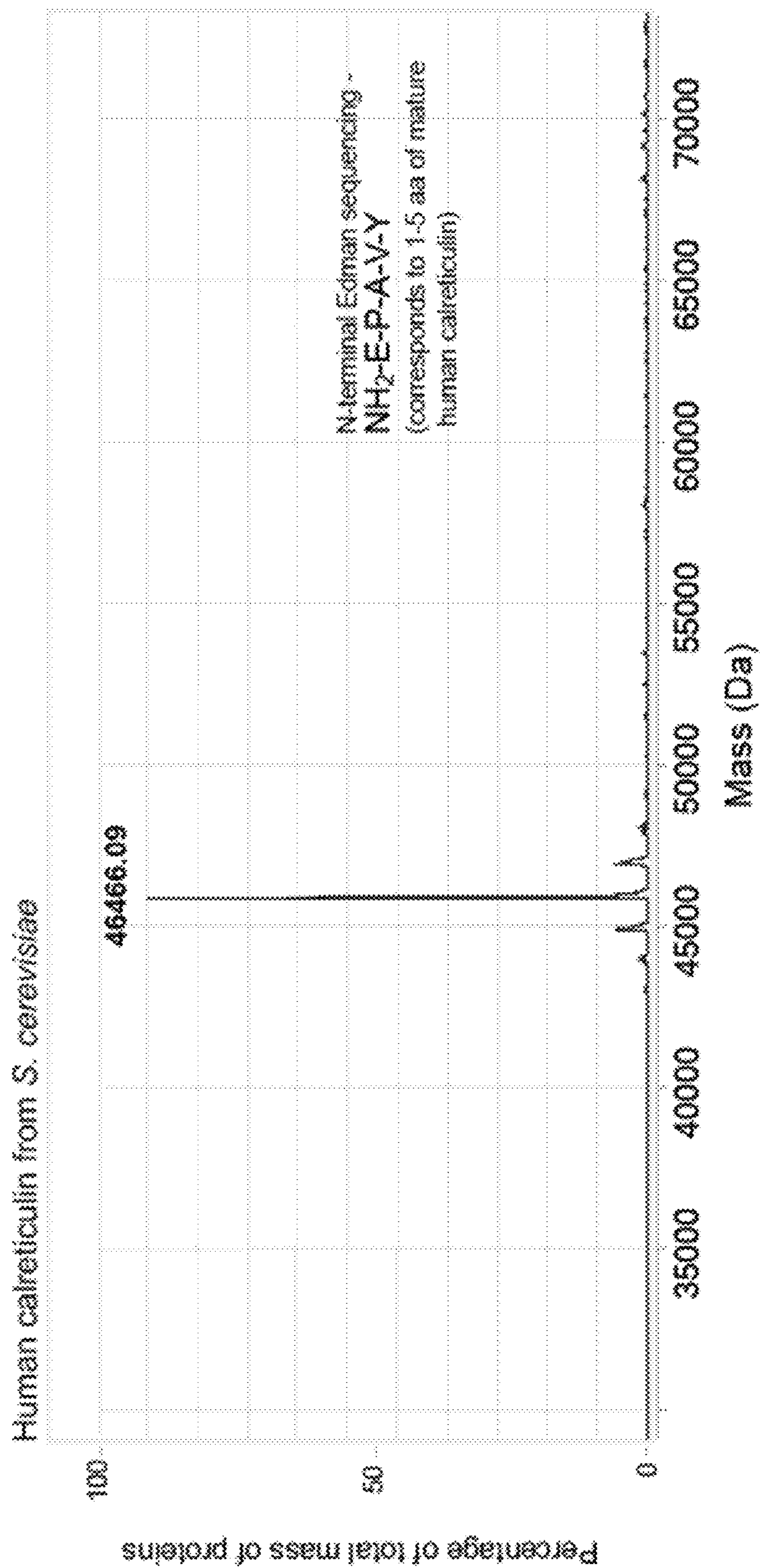


FIGURE 13

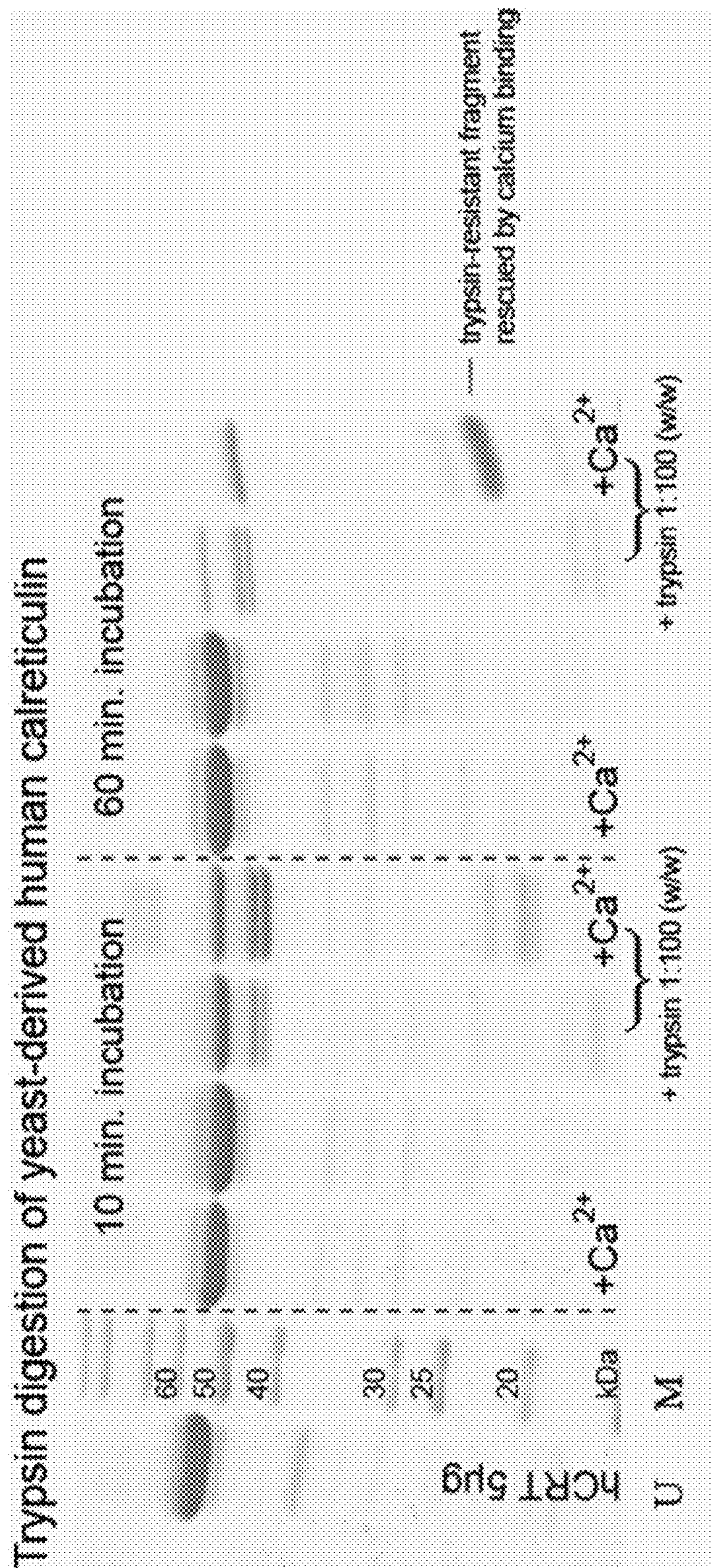


FIGURE 14

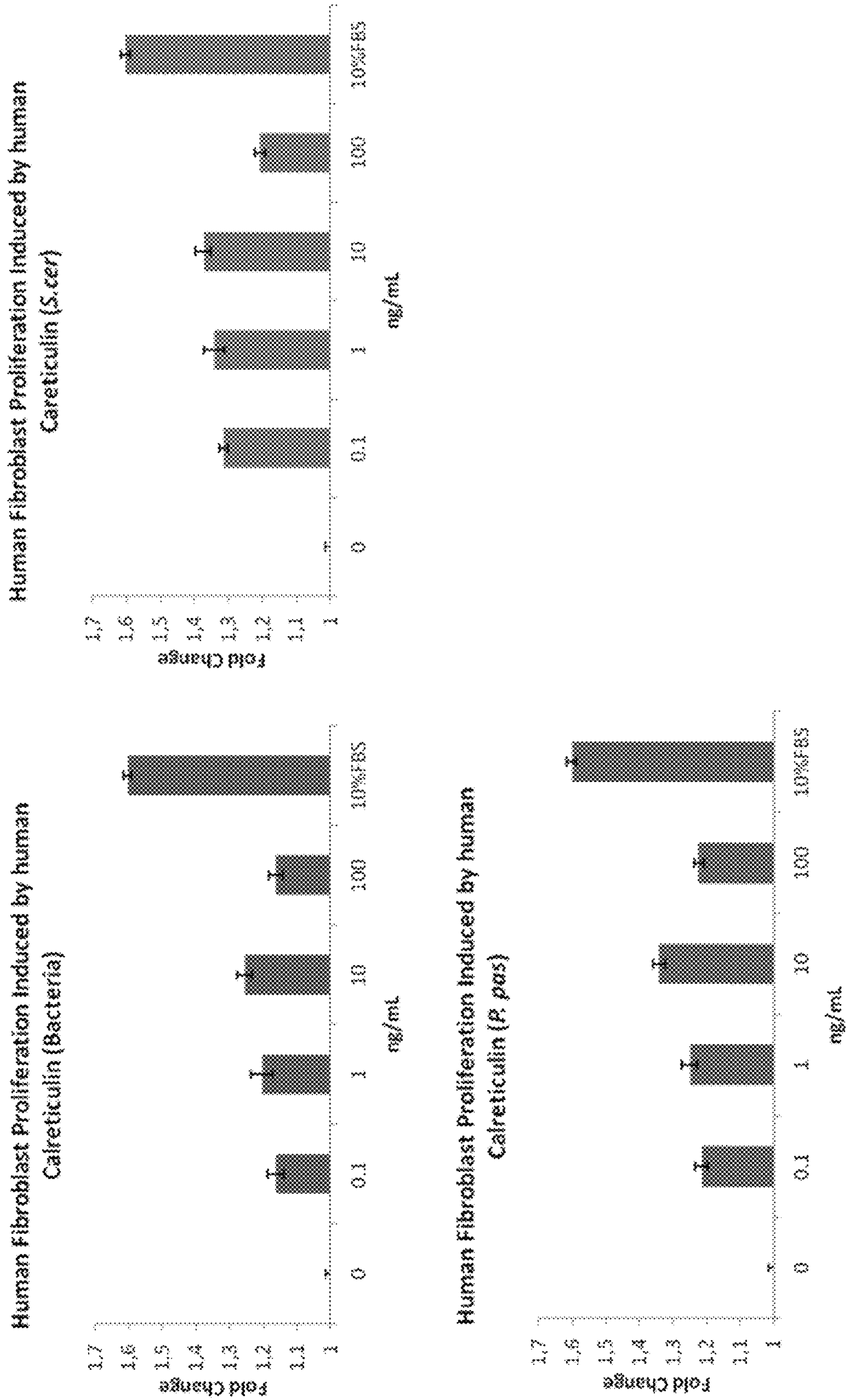


FIGURE 15

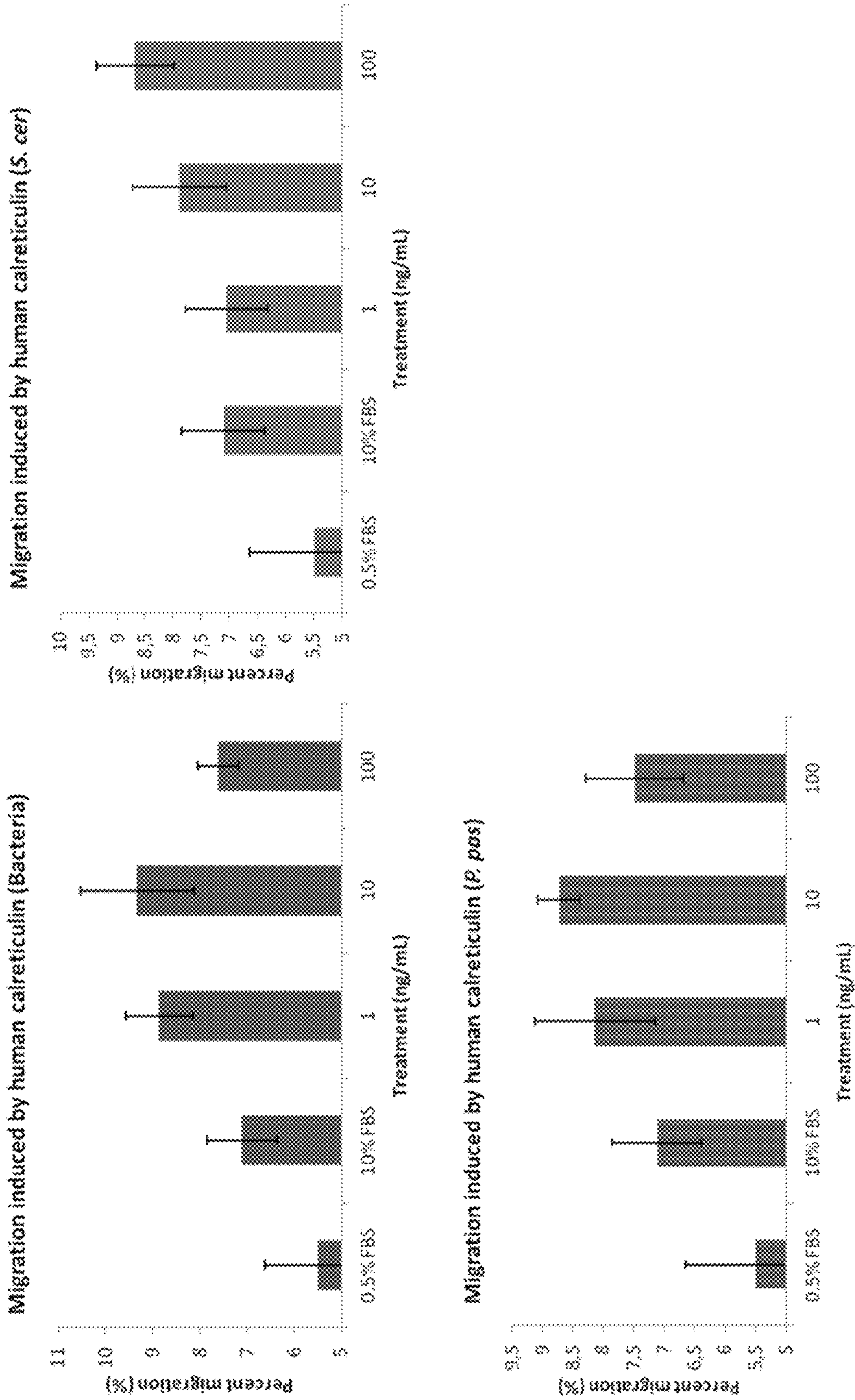


FIGURE 16

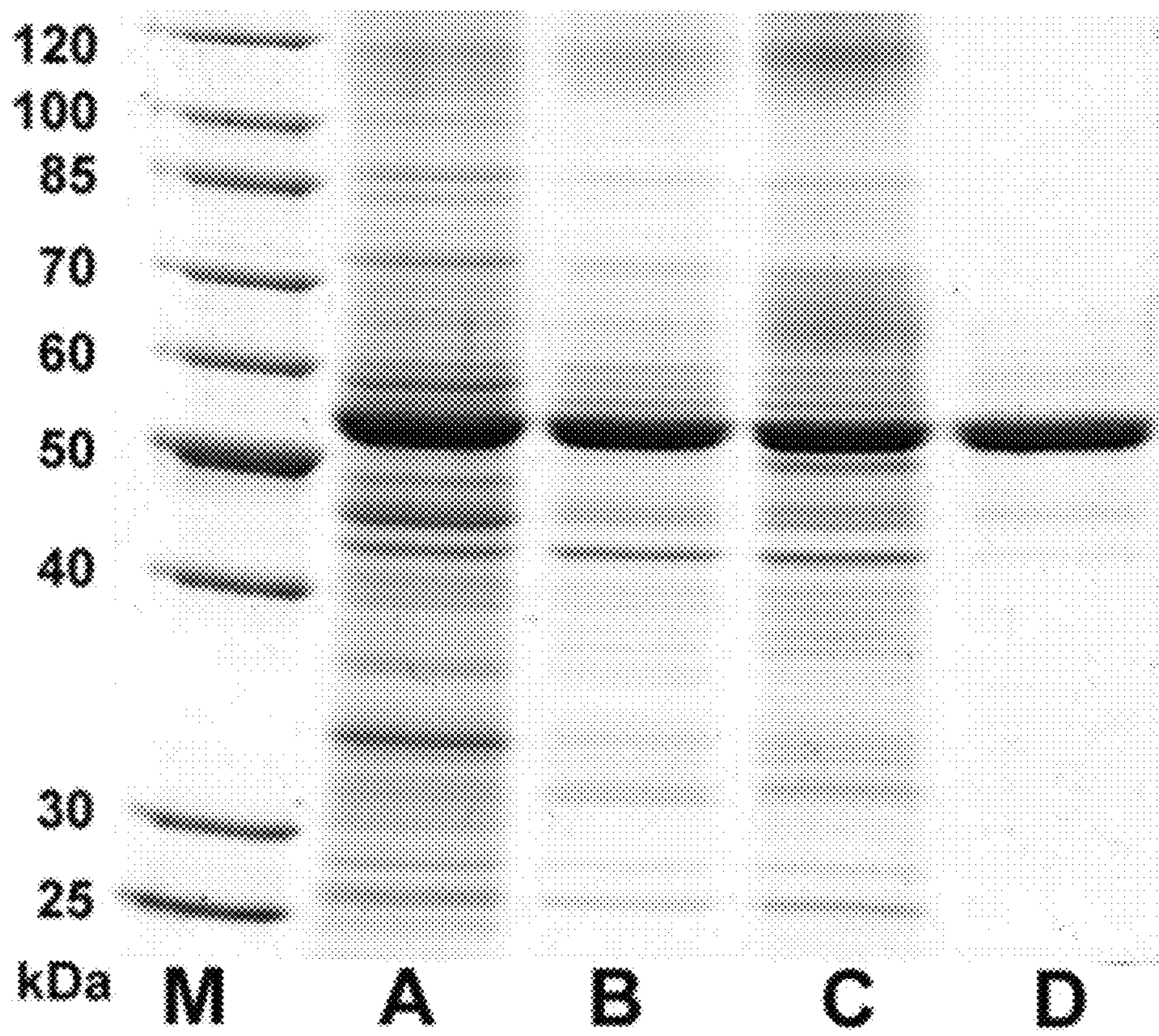


FIGURE 17

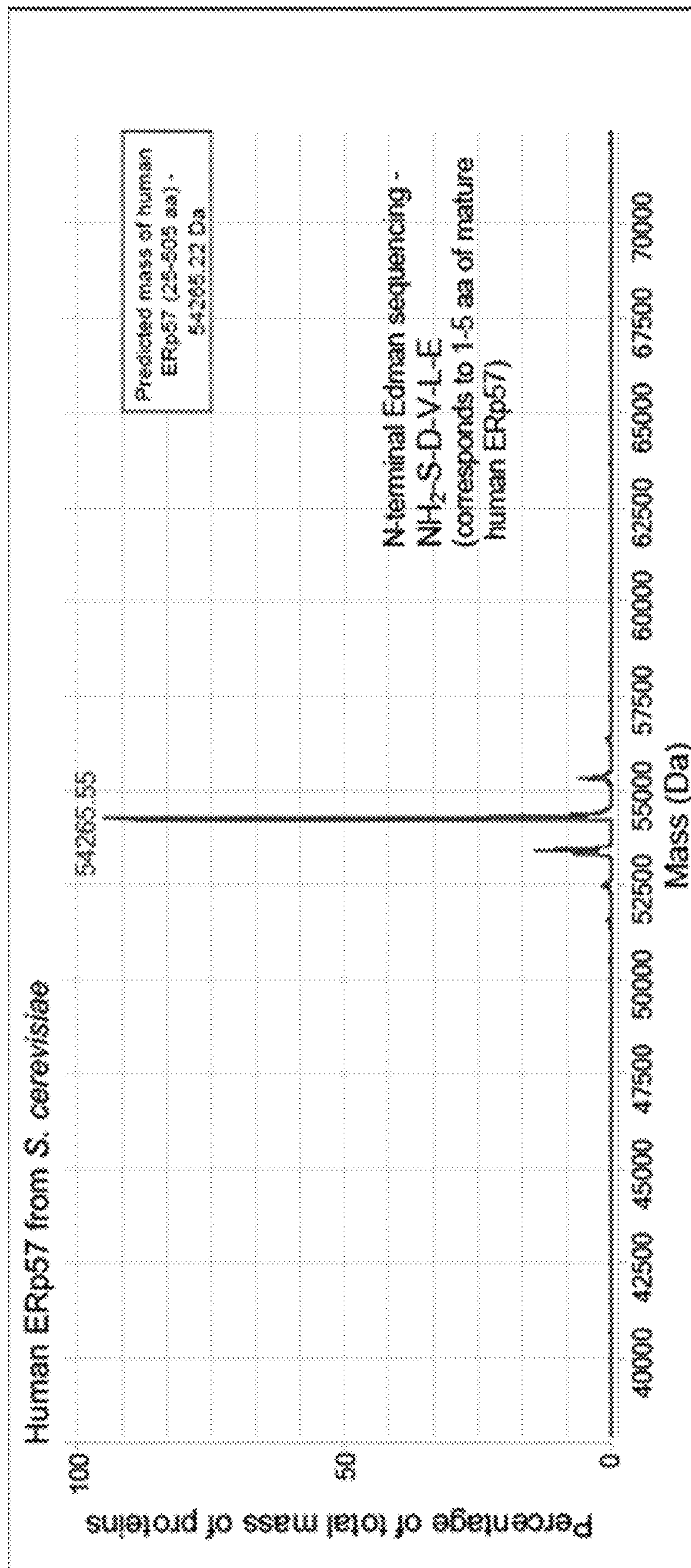


FIGURE 18

Protein PDIA3_Human.....P30101
 Mw 56782Da 505 aa. SEQ ID NO: 3

Without signal peptide
 Mw 54265Da 481 aa.

Protein is without signal
 Sequence of 24 aa length

1 MRLKRLALEP GVALLLAAR LAAASDVLEL TDNFESRIS DTGSAGMLV

start - End	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Sequence
25 - 38	847.2700	1638.7882	1638.7373	0.0509	0	A.SDVLELTDNFESR.I (LOSS SCORE 52)
25 - 38	820.4300	1638.8454	1638.7373	0.1081	0	A.SDVLELTDNFESR.I (LOSS SCORE 104)
38 - 38	820.4300	1638.8454	1638.7373	0.1081	0	A.SDVLELTDNFESR.I (LOSS SCORE 108)
25 - 38	820.4500	1638.8854	1638.7373	0.1481	0	A.SDVLELTDNFESR.I (LOSS SCORE 114)

The peptide begins not from lysine or arginine.
 This indicates that it is N-terminal residue.

FIGURE 19

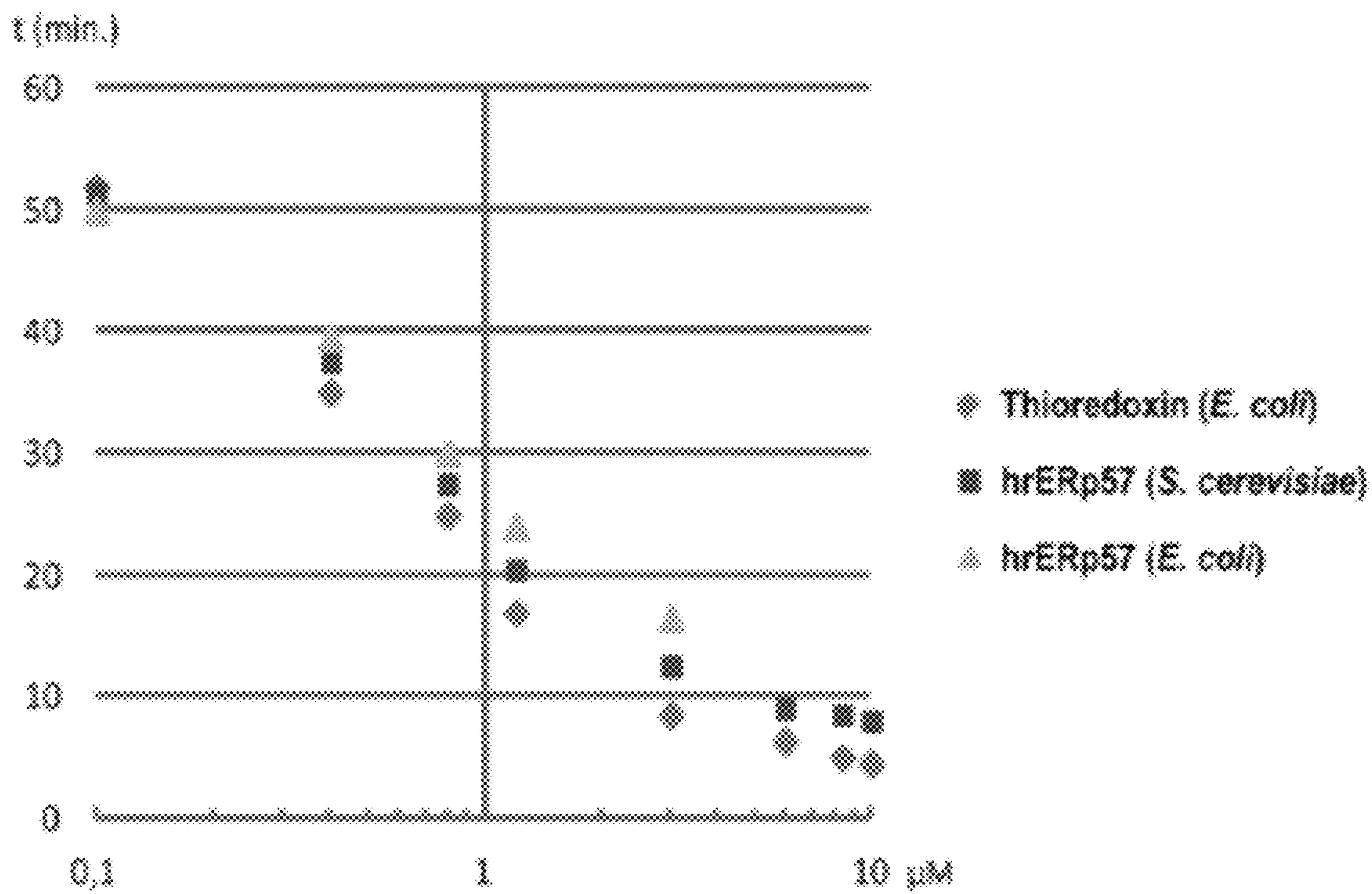
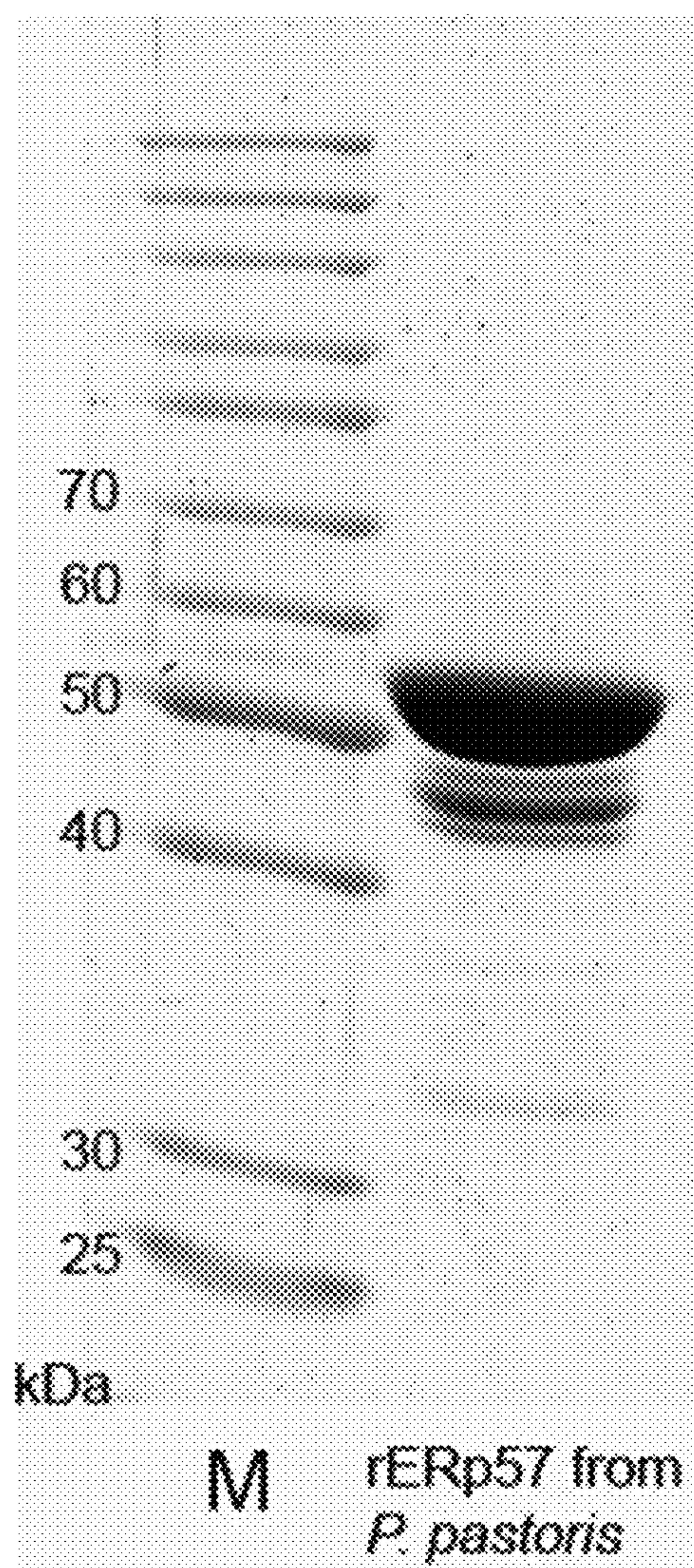


FIGURE 20



**GENERATION OF NATIVE RECOMBINANT
SECRETED HUMAN ENDOPLASMIC
RETICULUM CHAPERONES BY USING
THEIR NATIVE SIGNAL SEQUENCES IN
YEAST EXPRESSION SYSTEMS**

RELATED APPLICATIONS

This application is a Continuation of, and claims, priority under 35 U.S.C. §120 to, International Application No. PCT/US2013/049843, filed Jul. 10, 2013, and claims priority therethrough under 35 U.S.C. §119 to U.S. Provisional Application No. 61/670,768 filed Jul. 12, 2012, the entireties of which are incorporated by reference herein.

BACKGROUND

Endoplasmic reticulum (ER) chaperones are multifunctional proteins involved in a variety of biological processes such as protein folding and quality control in the ER (Hebert et al., 1995; Zhang et al., 1997; Braakman & van Anken, 2000; High et al., 2000; Bedard et al., 2005; Benyair et al., 2011; Braakman & Bulleid, 2011), unfolded protein response (Spear & Ng, 2001; Ma & Hendershot, 2004; Malhotra & Kaufman, 2007; Groenendyk et al., 2010; Chakrabarti et al., 2011), MHC class I antigen processing (Maffei et al., 1997; Nicchitta & Reed, 2000; Zhang & Williams, 2006; Wearsch & Cresswell, 2009), as well as other important functions these proteins play outside of the ER (Panayi & Corrigan, 2006; Gonzalez-Gronow et al. 2009; Gold et al., 2010; Ni et al., 2011; Peters & Raghavan, 2011; Turano et al., 2011). The role of ER chaperones in various human diseases seems especially important. There are growing amounts of data demonstrating involvement of particular ER chaperones in many pathological processes. For example, ER chaperone GRP78/BiP appears to be involved in cancer progression (Li & Lee, 2006; Lee, 2007; Luo & Lee, 2012), autoimmune inflammation and tissue damage (Panayi & Corrigan, 2006; Morito & Nagata, 2012) and rheumatoid arthritis (Corrigan et al., 2001; Yoo et al., 2012). Another ER chaperone calreticulin plays an important role in activating the anti-tumor response needed in chemotherapy or various other cancer treatment strategies (Chaput et al., 2007; Obeid et al., 2007; Wemeau et al., 2010) and is also associated with the healing processes of cutaneous wounds (Nanney et al., 2008). Other ER chaperones have also been implicated in disease related processes, such as prion diseases in the case of chaperone GRP58/ERp57 (Hetz et al., 2005). These recent findings suggest possible application of ER chaperones in therapeutic trials and development of new pharmaceuticals. Therefore, the growing demand of human ER chaperone protein products could be expected in the near future.

Native human ER chaperone proteins can be purified from various tissues, e.g. calreticulin has been purified from human placenta (Houen & Koch, 1994), however human tissues are not a sufficient source of these proteins for large scale clinical trials. The recombinant protein expression technologies should be considered for efficient and safe production of these proteins. Furthermore, it is desirable that the recombinant proteins for clinical trials should correspond to native analogs insofar as possible.

Currently most recombinant human ER chaperones are produced in bacterial host *Escherichia coli* (Rokeach et al., 1991; Baksh et al., 1992; Antoniou et al., 2002) and such products are commercially available (Abcam products ab78432, ab91577 and ab92937, 2012; StressMarq product

SPR-119B, 2012; USBiological products B1770-01, C1036-02L1 and E2291-75E, 2012). However, *E. coli* and other prokaryotes do not possess the ER, Golgi apparatus and other organelles of the eukaryotic secretion pathway, therefore it is uncertain that human ER proteins produced in bacteria will be correctly folded and possess all the same functions as the native protein analogues. Yeast is an attractive host for the production of the ER chaperones and other complex secreted human proteins, because this unicellular eukaryotic microorganism has eukaryotic features including a secretory pathway leading to correct protein processing and post-translational modifications (Mattanovich et al., 2012). Many attempts have being made to generate recombinant secreted human proteins in yeast (Damasceno et al., 2012; Hou et al., 2012) as such expression system facilitates purification and downstream processing of the protein product and the secreted proteins often are biologically active. Regarding generation of the secreted human ER chaperone proteins in yeast, several techniques may be used. All these approaches include use of the conventional yeast protein secretion signal fused to the sequence of processed mature human ER protein. The protein product generated in this way has several non-native amino acids on the N-terminus and the effect of this manipulation to biological activity of the prepared proteins is unclear. The only known example of yeast-expressed secreted full-length recombinant mammalian ER chaperone described in the literature so far is generation of recombinant rabbit calreticulin in yeast *Pichia pastoris* (Andrin et al., 2000).

SUMMARY OF THE INVENTION

No attempts to use native signal sequences of human ER chaperones in microbial hosts to produce correctly processed final native recombinant ER chaperone products in the same way as in the native analogs in human cells are known. Use of the native signal sequences of several intracellular human ER chaperones for the secretion of correctly processed final products to the culture media in yeast *S. cerevisiae* and *P. pastoris* expression systems is described herein. Surprisingly, this approach enabled generation of large amounts of native recombinant human chaperones processed in the same way as in human cells, except that mature protein products were secreted into yeast culture medium. The invention also shows experimental data that confirm correct processing of the signal sequences of human ER chaperones in yeast cells and provides how to efficiently produce these proteins in secreted form.

The present invention provides methods for producing native recombinant human ER chaperones in yeast with a simple downstream purification procedure. These proteins do not have any added artificial amino acid sequences and are processed into the final products, which exactly correspond to analogue human proteins according to their predicted molecular weight. This is achieved by integrating several key factors, as described below, and is only true for human endoplasmic reticulum (ER) luminal proteins. The results show how the method functions by describing the procedure with three human ER proteins as examples.

The present invention encompasses methods for producing recombinant proteins. These methods including the steps of

- (a) transforming a yeast cell with a nucleotide sequence comprising the coding sequence for a native human ER chaperone protein signal sequence and a human ER chaperone protein;

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(b) culturing the yeast cell under conditions such that the human ER recombinant chaperone protein is expressed in secreted form; and

(c) extracting the human ER recombinant chaperone protein from the culture medium.

One embodiment of the method in accord with the present invention produces a human ER recombinant chaperone protein, where the human ER chaperone protein is a human ER luminal protein. The method includes the steps of culturing a yeast cell, the yeast cell having been transformed with a nucleotide sequence comprising the coding sequence for both a native human ER chaperone protein signal sequence and a human ER chaperone protein, under conditions to express the human ER recombinant chaperone protein in secreted form; and extracting the human ER recombinant chaperone protein from the culture medium.

Another embodiment of the method in accord with the present invention produces a human ER recombinant chaperone protein selected from the group consisting of BiP/GRP78, calreticulin, and ERp57. The method includes the steps of

(a) providing a yeast cell transformed with a nucleotide sequence comprising the coding sequence for both a native human ER chaperone protein signal sequence and a human ER chaperone protein;

(b) culturing the yeast cell under conditions such that the human ER recombinant chaperone protein is expressed in secreted form; and

(c) extracting at least one of BiP/GRP78, calreticulin, or ERp57 from the culture medium.

Methods accord with the present invention may yield up to about 100 mg/L of the desired protein and the yield may be further increased by optimization of yeast culturing conditions by conventional methods well known in the art.

Another embodiment of the method in accord with the present invention produces human ER recombinant chaperone proteins by

(a) providing a yeast cell transformed with a nucleotide sequence comprising the coding sequence for both a native human ER chaperone protein signal sequence and a human ER chaperone protein;

(b) culturing the yeast cell under conditions such that the human ER recombinant chaperone protein is expressed in secreted form; and

(c) extracting the human ER recombinant chaperone protein from the culture medium.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows SDS-PAGE (panel A) and Western blot (panel B) analysis of 40× concentrated culture media of yeast *S. cerevisiae* AH22 cells transformed with empty plasmid (lane 1) or producing human chaperones BiP, calreticulin and ERp57 (lanes 2, 3 and 4, respectively).

FIG. 2A shows ESI-MS of secreted recombinant human chaperones calreticulin. FIG. 2B shows ESI-MS of ERp57. FIG. 2C shows ESI-MS of GRP78/BiP purified from *S. cerevisiae*.

FIG. 3 shows SDS-PAGE of unconcentrated culture media (8 μl each) from selected *P. pastoris* GS115 strain multicopy transformants overexpressing secreted recombinant human BiP (panel A), calreticulin (panel B) and ERp57 (panel C). C indicates control (8 μl of unconcentrated culture media from *P. pastoris* GS115 strain transformed with empty vector pPIC3.5K without human gene and cultured in parallel to strains overexpressing human chaperones),

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whereas M—protein markers with known molecular weights indicated over the bands.

FIG. 4 shows purification of secreted recombinant human GRP78/BiP protein from yeast *S. cerevisiae* culture medium. Lanes represent protein molecular weight markers (M), yeast culture medium after human BiP expression in *S. cerevisiae* (A), the same yeast growth medium after microfiltration (B), the same medium after tangential ultrafiltration (C) and secreted recombinant human BiP protein purified by ATP-affinity chromatography from the same medium (D).

FIG. 5 shows location of the tryptic peptide mass fingerprinting of *S. cerevisiae*-secreted GRP78/BiP protein band by using MALDI-TOF/TOF tandem MS/MS (mass spectrometry) together with UPLC/MS^E method.

FIG. 6 shows the results of N-terminal sequencing by Edman degradation and ESI-MS of a whole molecule of recombinant human GRP78/BiP secreted from *S. cerevisiae* and *P. pastoris*, respectively.

FIG. 7 shows partial proteolysis of recombinant BiP protein purified from *S. cerevisiae* (panel A) and *P. pastoris* (panel B).

FIG. 8 shows ATPase activity test using recombinant BiP proteins expressed in bacteria and yeasts.

FIG. 9 shows native PAGE of recombinant human BiP protein purified from *S. cerevisiae*.

FIG. 10 shows SDS-PAGE analysis of yeast culture media and purified recombinant human calreticulin samples from *P. pastoris* and *S. cerevisiae*, respectively.

FIG. 11 shows tryptic peptide mass fingerprinting of *S. cerevisiae*-expressed protein, which confirmed that purified secreted protein represents human calreticulin with correctly processed N-terminal amino acid sequence.

FIG. 12 shows ESI-MS and N-terminal Edman sequencing of secreted recombinant human calreticulin purified from *P. pastoris* and *S. cerevisiae*.

FIG. 13 shows trypsin digestion of recombinant human calreticulin purified from *P. pastoris* culture medium that confirms correct folding of yeast-secreted human protein.

FIG. 14 shows data on human fibroblast proliferation induced by recombinant calreticulin proteins derived from bacteria and yeasts.

FIG. 15 shows data of wound healing scratch plate assay. Human fibroblast migration was induced by recombinant calreticulin proteins derived from bacteria or yeasts.

FIG. 16 shows purification of secreted recombinant human ERp57 protein from yeast culture medium. Lanes represent protein molecular weight markers (M), crude yeast growth medium after human ERp57 expression in *S. cerevisiae* (A), the same yeast growth medium after microfiltration (B), the same medium after tangential ultrafiltration (C) and secreted recombinant human ERp57 protein purified from the same medium by one-step affinity chromatography using heparin Sepharose (D).

FIG. 17 shows ESI-MS and N-terminal Edman sequencing of secreted recombinant human ERp57 purified from *S. cerevisiae*.

FIG. 18 shows identification of protein N-terminus by tryptic peptide mass fingerprinting of secreted recombinant human chaperone ERp57 (PDIA3) purified from *S. cerevisiae*.

FIG. 19 shows thiol-dependent catalytic activity of yeast-derived recombinant human ERp57 protein assayed with the insulin precipitation method.

FIG. 20 shows SDS-PAGE of secreted recombinant human ERp57 purified from *P. pastoris*.

DETAILED DESCRIPTION

The invention encompasses yeast expression systems for the synthesis of native recombinant secreted human ER chaperone proteins in yeast cells. Yeasts are unicellular eukaryotic microorganisms capable of performing eukaryotic processing on the expressed polypeptides. Since yeast represent eukaryotes, their intracellular environment is more suitable for a correct folding of eukaryotic proteins including human cell proteins. Yeast-derived heterologous proteins are free of toxic contaminations and are excellent tools for developing vaccines, diagnostics or biopharmaceuticals. The yeast *Saccharomyces cerevisiae* is acknowledged as GRAS (generally regarded as safe) organism. The mostly native recombinant human or virus proteins generated in yeast possess similar properties as native proteins from human cells and are superior over their analogues expressed in bacteria. The growing demand for various recombinant proteins of high quality necessitates better and more efficient expression systems, even for the proteins with well-established production protocols. Native human proteins for various purposes are often purified from various human cells, as recombinant protein is synthesized and purified from *Escherichia coli* using various tags. The present invention demonstrated that yeast was by far a superior host for expression and purification of native recombinant human proteins.

ER chaperones are multifunctional proteins involved in a variety of biological processes such as protein folding and quality control in the ER, unfolded protein response, MHC class I antigen processing, and other important functions these proteins play outside of the ER. The role of ER chaperones in various human diseases appears especially important, with data demonstrating involvement of particular ER chaperones in many pathological processes. These recent findings suggest possible application of ER chaperones in therapeutic trials and development of new pharmaceuticals, along with fundamental and applied studies. Recombinant protein expression technologies provide efficient and safe production of these proteins. Examples provided here include, but are not limited to, GRP78/BiP, calreticulin and GRP58/ERp57 that are produced in yeast cells. The resultant proteins produced by the inventive method are tag-free. No tags were used for purification of these recombinant proteins; however, proteins could be His-tagged as well. The resultant proteins produced by the inventive method were correctly processed in yeast cell and the final protein product was composed from exactly the same amino acid sequence as in human cells. Non-native modifications were not present in the resultant recombinant products. The oligomeric state of the resultant recombinant proteins corresponded to that of native human chaperone proteins isolated from human tissues. The resultant recombinant proteins produced by the inventive method were fully active and were stable.

Three human ER proteins were used as examples in the disclosed procedure. Human genes HSPA5 (SEQ ID NO:4), CALR (SEQ ID NO:5), and PDIA3 (SEQ ID NO:6) (GeneBank id. no. respectively AF216292, M84739 and U42068), coding human ER chaperones, BiP/GRP78 (SEQ ID NO:1), calreticulin (SEQ ID NO:2), and ERp57 (SEQ ID NO:3), were cloned from commercial human liver cDNA library (Clontech, USA). The cDNA of the human genes was cloned intact, without any changes to the sequences

coding signal sequences, ER retention signals. The functional parts of the proteins were not removed, modified, or replaced with any homologous sequences from genes of the yeast or any other species. After nucleotide sequence analysis, human genes HSPA5, CALR, and PDIA3 were cloned into the yeast expression vector pFDC under control of yeast PGK1 gene promoter as described (Ciplys et al., 2011), resulting in three recombinant plasmids pFDC-BiP, pFDC-CALR, and pFDC-ERp57. pFDC-BiP, pFDC-CALR and pFDC-ERp57 yeast expression vectors, carrying human genes, were used for transformation of *S. cerevisiae* strain AH22 MATa (leu2 his4). Yeast transformation and subsequent selection of transformants were carried out exactly as described (Ciplys et al, 2011). After cultivation of yeast cells carrying recombinant plasmids in YEPD medium (yeast extract 1%, peptone 2%, dextrose 2%), proteins of yeast cells and growth medium were analyzed. Surprisingly, recombinant human chaperones were not only found in membrane protein fractions of the yeast cells, as previously described (Ciplys et al, 2011), but were also found in fairly large amounts in the growth medium, where they constituted up to about 50-60% of all proteins, as shown in FIG. 1.

About 1 L of culture medium contained 40 mg-50 mg secreted human calreticulin protein and 10 mg-15 mg of BiP/GRP78 and ERp57 proteins. The identity of the proteins was confirmed by Western blot analysis using specific antibodies against each human chaperone (FIG. 1 (panel A): lane 2—rabbit polyclonal antibodies against human BiP/GRP78 (Ab21685, Abcam, UK); lane 3—mouse monoclonal antibodies against human calreticulin (Ab22683, Abcam, UK); and lane 4—mouse monoclonal antibodies against human ERp57 (Ab13506, Abcam, UK). This observed phenomenon of high-level secretion of human ER chaperones using their whole cDNA nucleotide sequences without any changes in yeast was never described previously.

To further analyze the process, human HSPA5, CALR, and PDIA3 genes were cloned into the yeast expression vectors, similar to pFDC, but under different promoters of other yeast genes, ADH2, TDH2, TPI1, TEF, and ENO2. For expression of human BiP, calreticulin, and ERp57 proteins, different yeast *S. cerevisiae* strains were used (8188c, AH-214u, AH-214u Δ pep4). The results obtained using different promoters and strains were very similar to those shown in FIG. 1. This demonstrated that secretion of native amino acid sequence human chaperones into the yeast growth media was not associated with use of certain promoters or strains for their synthesis, but rather was a conjunction of different properties of the yeast cell: (i) ability to recognize signal sequence of human proteins and translocate them into the ER, but (ii) inability to retain human chaperones in their destined cell compartment. These properties were not discovered and/or utilized for production of native recombinant human proteins in yeast.

It should be noted that signal peptides of human chaperones actually are not secretion signal amino acid sequences, because they are used only for direction of native human proteins to the ER. When signal sequences are cleaved, chaperones in human cells are retained in the ER. Even though in some cases native human chaperones were shown to be directed to the cell surface, they were not secreted outside the cell. In fact, human ER chaperones are known as intracellular proteins. Therefore, here we for the first time show capabilities of yeast cells to secrete intracellular human proteins using their native signal sequences for intracellular processing and transfer of mature proteins inside the cell. It is known that some secreted human proteins may be secreted in yeast cells using their native

secretion signal sequences (Hitzeman et al, 1983; Barr et al, 1992). This process was patented in 1988 using human interferons as examples (Hitzeman and Leung, 1988) and also used in 1994 for patenting expression of human serum albumin using its native secretion signal sequence in *P. pastoris* (Prevatt and Sreekrishna, 1994). However, in the most cases *S. cerevisiae* α -MF prepro signal sequence was used for the secretion of other heterologous proteins in yeast, because native secretion signal sequences were less efficient (Cereghino and Cregg, 2000). Our findings are different from previous observations that native signal sequences may drive secretion of secreted human proteins in yeast. It may

ER signal sequences of human chaperone proteins are recognized and correctly processed in yeast cells, and this allows translocation of recombinant proteins into the ER following unexpected secretion outside the yeast cell. To check for possible modifications of secreted protein products we performed electrospray mass spectrometry (ESI-MS) analysis of a whole protein molecule to determine the exact molecular masses of yeast-secreted human ER chaperones. Results of mass spectrometry are given in Table 1 and FIG. 2A-2C; Table 1 indicates the exact determined molecular masses of the products and predicted molecular weights.

TABLE 1

N-terminal sequence, predicted and determined molecular weight of secreted recombinant human chaperones purified from <i>S. cerevisiae</i> .					
Protein	N-terminal sequence of the protein with indicated signal peptide ¹	Predicted ² molecular weight of whole protein	Predicted ² molecular weight of protein without signal sequence	Results of mass spectrometry of recombinant secreted human chaperones	N-terminal Edman sequencing of recombinant secreted human chaperones
BiP	MKLSLVAAME LLLSAARAE EDKKEDVGTV...	72332.96	70478.57	70478.39	NH ₂ -EEEDK
Calreticulin	MLLSVPLLLG LLGLAVAEPA VYFKEQFLDG...	48141.56	46466.37	46466.09	NH ₂ -EPAVY
ERp57	MLRRLALPP GVALLLAAR LAAASDVLEL...	56782.39	54265.22	54265.55	NH ₂ -SDVLE

¹N-terminal sequences with indicated signal peptides were taken from UniProtKB database, reviewed entries P11021 (GRP78_HUMAN), P27797 (CALR_HUMAN) and P30101 (PDIA3_HUMAN) for BiP, calreticulin and ERp57, respectively. Sequences of cleaved signal peptides are highlighted, whereas mature protein sequences are indicated in bold.

²Predicted molecular weights for BiP, calreticulin and ERp57 were calculated using free software tools in the same UniProtKB database sources.

be expected that secreted human proteins will also be secreted in yeast cells using the same signal sequence. In contrast, the secretion of intracellular human proteins, such as ER-resident chaperones, is not expected for recombinantly expressed analogues in yeast. Moreover, the secretion level of human ER chaperones in yeast is unexpectedly high and allows efficient production of correctly processed recombinant products. Taken together, we present in principle new protocols for secretion of heterologous human proteins in the yeast cells.

All three secreted human chaperones were then purified and analyzed. Purification of secreted recombinant human chaperones from culture media was performed using standard procedures such as microfiltration, ultrafiltration, and one-step chromatography, and standard protocols. This simple purification procedure was sufficient to achieve over 90% purity of native recombinant human chaperones. Such simple and effective downstream purification procedure was another advantage of the disclosed expression system. N-terminal sequencing by Edman degradation was performed for identification and characterization of purified secreted proteins. Edman sequencing (performed using a service of AltaBioscience) of the chaperone products from *S. cerevisiae* in all three cases identified the five N-terminal amino acids and showed that they corresponded to mature native chaperone products from human cells (see Table 1: NH₂-EEEDK for GRP78/BiP; NH₂-EPAVY for calreticulin, and NH₂-SDVLE for ERp57). These results indicate that native

Mass spectrometry results of yeast *S. cerevisiae*-derived purified human BiP, calreticulin and ERp57 proteins showed the masses of 70478.39, 46466.09 and 54265.55, respectively, which exactly correspond to theoretically predicted masses of mature human proteins (FIG. 2A-2C and Table 1). It indicates two things: (i) recombinant secreted human BiP, calreticulin and ERp57 proteins are exactly the same polypeptides as mature human ER proteins (including predicted ER retention signals KDEL or QEDL on the C-termini of the proteins) and (ii) they have no yeast-derived modifications—a very important characteristic for recombinant proteins. Moreover, mass spectrometry analysis revealed that the proteins were highly pure (FIG. 2A-2C).

Mass spectrometry results together with N-terminal sequencing of the secreted recombinant human BiP, calreticulin, and ERp57 proteins clearly showed that all three human chaperones were correctly processed in yeast cells. In the eukaryotic cells signal peptides of maturing proteins are recognized by translocon machinery that ensures proper translocation of protein into the ER. After the polypeptides are translocated in the ER lumen signal peptides are cleaved by a signal peptide peptidase complex that is located inside the ER (Zimmermann et al, 2011). No data exists about compatibility of human and yeast translocon machinery or signal peptide peptidase complex. The data disclosed herein show that specificity of these complexes from both species was generally the same, thus the signal sequence of maturing human chaperone in yeast cells was correctly recog-

nized by yeast proteins, resulting in proper cleavage of signal peptide following successful translocation of human protein into the ER. This phenomenon was never employed for the secreted production of intracellular recombinant human proteins in yeast cells. As mentioned, for secretion of recombinant proteins in various yeast species, the yeast secretion signal sequences are fused to target proteins resulting in several additional non-native amino acids adhered to the protein after secretion. The disclosed method not only simplified cloning of human genes for expression in yeast cells, but also ensured that recombinant human protein has exactly the same composition of amino acids as in human cells.

Thus, the identification of the N-termini of recombinant human BiP, calreticulin and ERp57 confirmed the results of mass spectrometry, namely, that secreted human chaperones purified from yeast *S. cerevisiae* had intact native amino acid sequences. ER retrieval sequences of all three proteins were also intact (KDEL amino acids in BiP and calreticulin, and QEDL in ERp57). Retrieval machinery of eukaryotic cells recognizes proteins that possess retrieval signals and retains them in the ER lumen (Capitani & Sallese, 2009). This indicated that the recombinant human chaperones were secreted from *S. cerevisiae* despite the presence of the ER retrieval signal. It raises question about reasons of secretion of human proteins by yeast cells and about retention of proteins in the ER in general. A leaky retention signal was described previously (Andrin et al., 2000; Hamilton & Gerngross, 2007) but was never utilized for production of secreted native recombinant proteins. Secretion of human ER proteins by *S. cerevisiae* cells could be explained by yeast preference for the HDEL rather than KDEL or QEDL signal for efficient retrieval of the ER-resident proteins (Dean and Pelham, 1990), but it was not the reason in this case, because replacement of KDEL or QEDL with the HDEL sequence did not suppress the secretion of BiP, calreticulin and ERp57 (our unpublished data). Also, overload of the yeast ER retrieval machinery can be omitted as the reason for secretion of human ER proteins, because overexpression of yeast Kar2 protein with native HDEL ER retrieval sequence using the same pFDC vector did not lead to the secretion of this protein (our unpublished data). Moreover, human PDI, which is homologue of human ERp57 and yeast PDI proteins and contains KDEL ER retrieval sequence, was also expressed using the same pFDC vector, and in this case secretion of recombinant protein was not observed (our unpublished data). These experiments indicate that retention of ER luminal proteins is complicated and still unsolved mechanism, which does not strictly depend on HDEL/KDEL retrieval mechanism. Our finding, secretion of human ER chaperones by yeast cells, could serve as a convenient model for studying this phenomenon. On the other hand, this process may be exploited for efficient production of native recombinant human ER proteins in yeasts.

Taking together, these results clearly demonstrated that a newly discovered phenomenon of the ability of yeast cells to recognize and correctly process signal sequence of human ER proteins, with subsequent secretion of mature products to the culture media, provides new opportunities for synthesis of recombinant ER chaperones in yeast. The inventive findings allow synthesis of secreted native recombinant human chaperones with a simple downstream purification procedure. Moreover, recombinant human proteins passed the protein secretion pathway of yeast cells, which might ensure their proper maturation.

The present invention also encompasses other yeast expression systems including, but not limited to, *P. pastoris*. The same cDNA constructs encoding full sequences of human chaperones with native signal sequences for the secretion of protein products were used. Secretion of all three protein products was shown (see the Examples). The expression of GRP78/BiP and calreticulin (Examples 1 and 2) was more efficient in the *P. pastoris* system, which can be used to considerably enhance the product yields. SDS-PAGE images of *P. pastoris* culture media samples for both BiP and CRT proteins illustrate efficiency of secretion in this host (FIG. 3 (panels A and B)). Initially, the amount of secreted ERp57 in *P. pastoris* was lower than in *S. cerevisiae*. However, after optimization of cultivation conditions of *P. pastoris* culture, the amount of secreted human ERp57 exceeded that of *S. cerevisiae* cells (FIG. 3 (panel C)). Optimized protocol for the expression of ERp57 in *P. pastoris* is provided in the Example 3. After secretion to the culture media, human BiP, calreticulin and ERp57 proteins were purified from *P. pastoris* by using the same methods as in the case of expression in *S. cerevisiae*. The properties of purified products were similar to those of analogous proteins derived from *S. cerevisiae* cells (more detailed description of the experiments is given in the Examples). Therefore, our invention refers to generation of human ER chaperones in yeasts in general, rather to the expression in one yeast species.

Finally, we claim for the generation of biologically active human ER chaperones in yeast. Here we show that all three yeast-derived human proteins BiP, calreticulin and ERp57 are correctly folded and possess biological activity. GRP78/BiP protein produced in accord with the disclosed invention showed correct folding and activity of the product. Yeast-expressed BiP protein bound ATP, and this protected a ~60 kDa fragment from proteolysis by proteinase K (FIG. 7). Similar data was previously used to demonstrate correct folding of *E. coli* expressed analogous protein. For activity of yeast-expressed protein, an ATP-ase activity test of GRP78/BiP was used, also using an *E. coli* expressed analog as parallel control. As shown in FIG. 8, there was three-fold higher yeast-expressed secreted BiP chaperone activity than that of commercial *E. coli* product (~6.3 μ M versus ~2.1 μ M of hydrolysed ATP by the same amount of protein in the same time). ATP binding and protection of ~60 kDa domain of *P. pastoris*-derived BiP was similar to that from *S. cerevisiae* (FIG. 7 (panel B)). The ATP-ase activity of *P. pastoris*-expressed BiP was also measured and showed similar results as for *S. cerevisiae*-expressed analog (FIG. 8, the average for *P. pastoris*-expressed BiP was slightly higher than for *S. cerevisiae* analog, but within the range of error) with about three fold increase compared to *E. coli*-expressed product. This considerable difference demonstrated an advantage of yeast-expressed products. Partial digestion of calreticulin with trypsin (Corbett et al., 2000; Højrup et al., 2001) suggested correct folding and Ca²⁺ binding of yeast-expressed hCRT, as it is shown in Example 2. Furthermore, in vitro assay for cellular proliferation (Nanney et al., 2008; Greives et al., 2012) showed slightly but significantly higher induction of human fibroblast proliferation by both *S. cerevisiae*- and *P. pastoris*-derived human calreticulin compared to the same protein purified from bacteria *E. coli* (Table 2 and FIG. 14). Moreover, wound healing scratch plate assay showed that both *E. coli*- and yeast-expressed recombinant calreticulin induce migration of human fibroblasts at the similar extent within the range of error (Table 3 and FIG. 15). Taken together, the data of yeast-expressed human calreticulin demonstrated that recombinant product

possesses at least the same biological activity as recombinant calreticulin derived from bacteria. It should be noted that the same *E. coli*-expressed calreticulin protein, used as a control in these assays, previously has shown profound effects on the process of wound healing in vivo by causing a dose-dependent increase in epithelial migration and granulation tissue formation in both murine and porcine normal and impaired animal models of skin injury (Gold et al., 2006; Greives et al., 2012). Therefore, it could be expected that the yeast-derived secreted human calreticulin may also be successfully used for wound healing in vivo. Regarding ERp57, the data showed thiol-dependent reductase activity of ERp57 chaperone. Comparing *S. cerevisiae*-expressed ERp57 chaperone product with the same commercially available recombinant chaperone expressed in bacteria *E. coli*, the data demonstrated that yeast-expressed protein was slightly but considerably more active than the same amount of ERp57 analog expressed in *E. coli* (FIG. 19). Therefore, the disclosed system could generate more active product than in *E. coli* and provides an efficient platform for the production of recombinant ER chaperones.

The invention will be further appreciated with reference to the following non-limiting examples.

Example 1

Generation of Native Recombinant Human GRP78/BiP Protein in Yeast Expression Systems

Human BiP/GRP78 is a major endoplasmic reticulum chaperone which plays a dual role in the ER by controlling protein folding, in order to prevent aggregation, and by regulating the signaling of the unfolded protein response (UPR). It also participates in many other important cellular processes, such as calcium homeostasis, apoptosis regulation and signal transduction. Recently, it has been shown that this protein is of importance in cancerous cells and it could potentially be used for therapeutic purposes. Also, growing body of evidence indicates GRP78 as a new therapeutic target for treatments of forebrain ischemia, Parkinson disease and retinal degeneration (Gorbatyuk and Gorbatyuk, 2013). In this study we present evidences, that yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* are perfect hosts for expression and purification of native recombinant human BiP/GRP78 protein. The newly discovered ability of the yeast cells to recognize and correctly process native signal sequence of human BiP/GRP78 protein consequently secreting it into the growth media, allows simple one-step purification of highly pure recombinant BiP/GRP78 protein with yields reaching 10 mg/L and 20 mg/L from *S. cerevisiae* and *P. pastoris* respectively. The data showed that it was fully intact and active protein without yeast derived modifications. Yeast-derived human BiP/GRP78 protein possesses ATPase activity, which 3-fold exceeds activity of *E. coli*-derived recombinant human BiP/GRP78.

cDNA encoding full-length human GRP78/BiP (gene HSPA5 Acc. no. AF216292) was amplified from commercial human adult liver cDNA library (Clontech) by PCR using specific oligonucleotide primers BiPF (gta tct aga aca atg aag ctc tcc ctg gtg g) and BiPR (cag tct aga cta caa ctc atc ttt ttc tgc tgt), digested with restriction endonuclease (RE) XbaI and cloned into yeast expression vectors pFDC (Čiplys et al., 2011) and pPIC3.5K (Intvitrogen) into RE sites XbaI and AvrII under control of *S. cerevisiae* PGK1 or *P. pastoris* AOX1 promoters, respectively. Cloned HSPA5 gene sequence (beginning from start codon ATG and ending with STOP codon TAG) was verified by DNA sequencing and

generated plasmids pFDC-BiP and pPIC3.5K-BiP were used for transformation of yeast *S. cerevisiae* and *P. pastoris*, respectively. *S. cerevisiae* transformants were selected by resistance to formaldehyde and harboured multicopy autonomously replicating plasmid pFDC-BiP, whereas multicopy *P. pastoris* transformants were selected by resistance to G418 according to standard procedure well known in the art and strains with the most efficient secretion of BiP protein were chosen for further experiments. Both yeasts were used for expression of the full length GRP78/BiP protein including native N-terminal signal peptide. Both *S. cerevisiae* and *P. pastoris* secreted GRP78/BiP protein product into the culture media. The secretion was more efficient in the selected *P. pastoris* clone. 40 times concentrated culture medium of yeast *S. cerevisiae* AH22 strain transformed with plasmid pFDC-BiP is shown in FIG. 1 (lanes 2), whereas 8 microliters of unconcentrated culture medium from the most efficient selected *P. pastoris* clone is shown in FIG. 3, panel A (lane rBiP; lane C represents control medium from *P. pastoris* transformed with empty vector pPIC3.5K without a human gene, whereas lane M—protein markers with indicated molecular weights).

After expression of recombinant human BiP, cells were separated from the medium by centrifugation and yeast growth medium was further prefiltered with subsequent microfiltration of secreted protein through 0.2 μ m filter. After microfiltration, proteins were concentrated and transferred into the binding buffer (20 mM HEPES, 50 mM NaCl, 10 mM MgCl₂, pH 7.5) through tangential ultrafiltration using cassettes with 50 kDa cut-off membranes. Further, proteins were mixed with 8AH-ATP-agarose (Jena Bioscience) equilibrated in the same buffer and incubated for 2-3 hours at 4° C. in batch format. Unbound proteins were removed by washing with 20 column volumes of binding buffer while bound proteins were eluted with equal column volume of elution buffer (20 mM HEPES, 50 mM NaCl, 10 mM MgCl₂, 5 mM ATP, pH 7.5). Elution fractions were analyzed by SDS-PAGE. Three subsequent elution fractions showed ~95% pure human GRP78/BiP protein. These fractions were pooled and dialysed against ATPase buffer (50 mM HEPES, 50 mM NaCl, 2 mM MgCl₂, 5 mM ATP, pH 6.8) or BiP storage buffer (20 mM Tris-HCl, 350 mM NaCl, 0.5 mM DTT, 10% glycerol, pH 8.0). Such purification procedure was enough to reach ~95% purity of secreted recombinant human BiP. Yields obtained were approx. 10 mg and 20 mg from 1 L culture medium in *S. cerevisiae* and *P. pastoris* expression systems, respectively.

FIG. 4 shows SDS-PAGE analysis of yeast culture media and purified recombinant human BiP sample from *S. cerevisiae*. A—yeast culture medium after human BiP expression in *S. cerevisiae* (20 \times concentrated medium supernatant); B—yeast growth medium after microfiltration; C—20 \times concentrated proteins from yeast growth medium after tangential ultrafiltration; D—3 μ g of secreted recombinant human BiP protein purified by ATP-affinity chromatography from *S. cerevisiae*. M—protein markers with molecular weights indicated at the left. SDS-PAGE analysis of human BiP purification from *P. pastoris* is not shown, but it is similar to that of *S. cerevisiae*.

The band of purified secreted GRP78/BiP protein was excised from SDS-PAA gel and identified by trypsin digestion and MALDI-TOF/TOF tandemic MS/MS (mass spectrometry) together with UPLC/MS^E method using a service of the Proteomics Centre at the Institute of Biochemistry (Vilnius, Lithuania). Tryptic peptide mass fingerprinting confirmed that purified secreted protein represents human GRP78/BiP, which was identified by both methods with a

high level of confidence and ~57% sequence coverage (FIG. 5). FIG. 5 shows identified peptides (indicated in bold) of GRP78_HUMAN protein (Acc. No. P11021 in UniProt KB database). We were not able to identify N-terminal tryptic peptide by this method, which corresponds to N-terminal sequence of native mature GRP78/BiP from human cells. However, search in a UniProtKB database using PLGS (ProteinLynx Global Service) search engine in UPLC/MS^E method identified a C-terminal human GRP78/BiP peptide (Y)GSAGPPPTGEEDTAEKDEL(-), which is underlined in FIG. 5. This demonstrated that secreted human BiP protein possesses intact C-terminal amino acid sequence including KDEL ER retention/retrieval signal. Further, the molecular mass of both *S. cerevisiae*- and *P. pastoris*-secreted BiP protein was measured by electrospray mass spectrometry (ESI-MS) using Agilent Q-TOF 6520 mass spectrometer. ESI-MS of a whole *S. cerevisiae*-derived recombinant GRP78/BiP protein molecule showed a molecular mass of ~70478 Da, which exactly corresponds to theoretically predicted mass of mature human GRP78/BiP (19-654 aa) (FIG. 6, upper panel). Whereas, *P. pastoris*-secreted BiP protein showed similar but slightly different molecular mass of 70482.74 Da (FIG. 6, lower panel), which was only 4 Da greater than the predicted mass of mature human GRP78/BiP (70478.57 Da). We suppose that this discrepancy may result from different buffers from which yeast-derived human BiP was taken for the analysis. In the case of *S. cerevisiae*-secreted BiP protein, we took a sample directly from ATPase buffer (50 mM HEPES, 50 mM NaCl, 2 mM MgCl₂, 5 mM ATP, pH 6.8), while *P. pastoris*-derived human GRP78/BiP was taken for the analysis from a BiP storage buffer (20 mM Tris-HCl, 350 mM NaCl, 0.5 mM DTT, 10% glycerol, pH 8.0) containing DTT. Possibly, increased molecular mass of *P. pastoris*-expressed BiP protein is determined by reduced cysteines in DTT containing sample.

Furthermore, N-terminal sequencing by Edman degradation confirmed that the first five N-terminal amino acids of the recombinant protein from both yeasts were NH₂-EEEDK (FIG. 6), which correspond the N-terminal sequence of mature human BiP protein after signal cleavage (Table 1). Taken together, these results indicated that native ER signal sequence of human BiP protein is recognized and correctly processed in yeast cells, and this allows translocation of recombinant protein into the ER following unexpected secretion outside the yeast cell. Also, the results proved that secreted human GRP78/BiP protein purified from the yeast cells does not carry any modifications.

GRP78/BiP protein produced in accord with the disclosed invention showed correct folding and activity of the product. Correct folding was assessed by partial proteolysis of recombinant BiP protein purified from *S. cerevisiae* and *P. pastoris* using a method described in Wei and Hendershot, 1995. The results are shown in FIG. 7 displaying partial proteolysis of BiP from *S. cerevisiae* (FIG. 7, panel A) and *P. pastoris* (FIG. 7, panel B): 5 µg of human BiP protein purified from yeast culture media was loaded onto each lane: 1—without proteinase; 2, 3 and 4—treated with 2 µg of proteinase K in the presence of 100 µM ATP (lanes 3) or 100 µM ADP (lanes 4) or in the absence of any of nucleotides (lanes 2). Reaction was performed in 65 µl volume in ATPase buffer (50 mM HEPES, pH=6.8, 50 mM NaCl, 2 mM MgCl₂) at 37° C. for 25 min., then stopped by adding 10 µl of 1 mg/ml PMSF and incubated on ice for 30 min. (according to Wei and Hendershot, 1995). Digested BiP protein was further analyzed by SDS-PAGE. M—molecular mass standards (Fermentas, Lithuania, SM0671). *S. cerevi-*

siae-expressed BiP protein bound ATP, and this protected a ~60 kDa fragment from proteolysis by proteinase K, whereas binding of ADP protected ~44 kDa fragment (FIG. 7, panel A). Similar data was previously used to demonstrate correct folding of both native mammalian (Kassenbrock and Kelly, 1989) and *E. coli*-expressed recombinant BiP proteins (Wei and Hendershot, 1995).

For activity of yeast-expressed protein, an ATP-ase activity test of GRP78/BiP was used, also using an *E. coli* expressed analog as parallel control. The results are shown in FIG. 8. BiP protein produced in *E. coli* was purchased from Nordic BioSite (Cat. No. SPR-119), whereas yeast-expressed proteins were purified in this study. Reactions were performed in 50 µl volumes as follows: 1 µg of recombinant BiP protein (or equal volume of buffer for negative control) with 20 mM KCl and 20 µM ATP in ATPase buffer (50 mM HEPES, pH=6.8, 50 mM NaCl, 2 mM MgCl₂) was incubated at 25° C. for 75 min. Concentration of the phosphate liberated from ATP was measured by spectrophotometer (TECAN Infinite® 200, wave length 620-650 nm) using Malachite Green Phosphate Assay Kit (Cayman Chemical) according to manufacturer' recommendations (detailed procedure of performed test is described in Bernal-Bayard et al., 2010). As it is shown in FIG. 8, there was three-fold higher yeast-expressed secreted BiP chaperone activity than that of commercial *E. coli* product (~6.3 µM versus ~2.1 µM of hydrolysed ATP by the same amount of protein in the same time). This considerable difference demonstrated an advantage of yeast-expressed products. ATP binding and protection of ~60 kDa domain of *P. pastoris*-derived BiP, as well as ~44 kDa fragment protected by ADP, was similar to that from *S. cerevisiae*-derived BiP (FIG. 7, panel B). The ATP-ase activity of *P. pastoris*-expressed BiP was also measured and showed similar results as for *S. cerevisiae*-expressed analog (FIG. 8, the average for *P. pastoris*-expressed BiP was slightly higher than for *S. cerevisiae* analog, but within the range of error) with about three fold increase compared to *E. coli*-expressed product.

To explain such considerable difference in activity between yeast- and bacteria-expressed human BiP proteins we performed further experiments including test of BiP oligomerization by using native PAGE. Native PAGE procedure with yeast-expressed BiP protein was performed according to the protocol of Freiden et al., 1992, which was used for the assessment of the oligomerization of both native mammalian dog BiP (Freiden et al., 1992) and of *E. coli*-expressed hamster BiP protein (Wei and Hendershot, 1995). The native PAGE with *S. cerevisiae*-derived human BiP protein is shown in FIG. 9. 5 µg of human BiP protein purified from *S. cerevisiae* culture medium was loaded onto gel lane, whereas 5 µg of BSA was used as a molecular weight marker for the native PAGE. Observed approximate molecular weights are indicated in FIG. 9. Native PAGE of *P. pastoris*-derived human BiP is not shown, but it revealed very similar results. This test demonstrated that yeast-derived secreted human BiP protein is present in predominantly monomeric form. In mammalian cells BiP exists as both monomer and dimer (Freiden et al., 1992; Wei and Hendershot, 1995). In contrast, *E. coli*-expressed BiP protein was found mostly in dimeric form, although some monomers and higher order oligomers were also present (Blond-Elguindi et al., 1993; Wei and Hendershot, 1995). Therefore, these differences in oligomeric state might be related to different enzymatic activities of recombinant human BiP proteins. Moreover, yeast-expressed recombinant BiP protein undergoes protein quality control throughout the yeast secretion pathway; meanwhile *E. coli*-synthe-

sized BiPs are purified by capturing all expressed BiP protein molecules independently of their folding state.

Example 2

Native Signal Peptide of Human Calreticulin Mediates Efficient Secretion of Correctly Processed Mature Recombinant Calreticulin in Yeast

Growing amount of data associates calreticulin with many different functions in subcellular locations outside the ER. Analysis of protein functions requires substantial amounts of correctly folded, biologically active protein. In this study we introduce yeast *Saccharomyces cerevisiae* and *Pichia pastoris* as perfect hosts for production of human calreticulin. Our data demonstrate that native signal peptide of human calreticulin protein is recognized and correctly processed in the yeast cells, which leads to protein secretion. Secretion allows simple one-step purification of recombinant calreticulin protein from yeast culture medium with the yields exceeding 30 and 100 mg/L in *S. cerevisiae* and *P. pastoris*, respectively. Analysis of yeast-expressed secreted recombinant human calreticulin revealed that it possesses native amino acid sequence as in human cells and non-native modifications are not present in the recombinant product. Furthermore, limited proteolysis with trypsin suggested that yeast-derived calreticulin is correctly folded Ca^{2+} binding protein. Finally, the recombinant secreted products appeared to be biologically active and induced cellular proliferation and migration of human fibroblasts in a wound healing scratch plate assay.

cDNA encoding full-length human calreticulin (Acc. no. M84739) was amplified from commercial human adult liver cDNA library (Clontech) by PCR using specific oligonucleotide primers CRTF (gta tct aga aca atg ctg cta tcc gtg ccg ttg) and CRTR (cag tct aga cta cag ctc gtc ctt ggc ctg), digested with restriction endonuclease (RE) XbaI and cloned into yeast expression vectors pFDC (Ciplys et al., 2011) and pPIC3.5K (Intvitrogen) into RE sites XbaI and AvrII under control of *S. cerevisiae* PGK1 or *P. pastoris* AOX1 promoters, respectively. Cloned CRT gene sequence (beginning from start codon ATG and ending with STOP codon TAG) was verified by DNA sequencing and generated plasmids pFDC-CRT and pPIC3.5K-CRT were used for transformation of yeast *S. cerevisiae* and *P. pastoris*, respectively. *S. cerevisiae* transformants were selected by resistance to formaldehyde and harboured multicopy autonomously replicating plasmid pFDC-CRT, whereas multicopy *P. pastoris* transformants were selected by resistance to G418 and strains with the most efficient secretion of CRT protein were chosen for further experiments. Both yeasts were used for expression of the full length CRT protein including native N-terminal signal peptide.

After expression of recombinant human calreticulin, cells were separated from the medium by centrifugation and yeast growth medium was further prefiltered with subsequent microfiltration of secreted protein through 0.2 μm filter. After microfiltration, proteins were concentrated and transferred into the binding buffer (20 mM Tris-HCl, pH 8.0) through tangential ultrafiltration using cassettes with 100 kDa cut-off membranes. Further, proteins were purified by ion-exchange chromatography on Sepharose Q. Such purification procedure was enough to reach up to 90% purity of secreted recombinant human calreticulin. Yields obtained were approx. 30 mg and 100 mg from 1 L culture medium in *S. cerevisiae* and *P. pastoris* expression systems, respectively.

FIG. 10 shows SDS-PAGE analysis of yeast culture media and purified recombinant human calreticulin samples. Upper panel—yeast culture media after CRT expression in *P. pastoris* (10 \times concentrated medium supernatant) and *S. cerevisiae* (30 \times concentrated medium supernatant). A—media from control yeast strains without CRT gene; B—media from yeast strains expressing human CRT. Lower panel—purified secreted recombinant human CRT protein (C lanes). M—protein molecular weight markers.

Tryptic peptide mass fingerprinting confirmed that purified secreted protein represents human calreticulin (FIG. 11, identified peptides are indicated in bold). Moreover, N-terminal tryptic peptide (EPAVYFK) was identified, which corresponds to N-terminal sequence of native mature calreticulin from human cells. Further, an important result was obtained by ESI-MS of a whole yeast-derived recombinant calreticulin protein molecule. Both *P. pastoris*- and *S. cerevisiae*-secreted CRT protein showed a molecular mass of ~ 46466 Da, which exactly corresponds to theoretically predicted mass of mature human CRT (18-417 aa) (FIG. 12). Furthermore, N-terminal sequencing by Edman degradation confirmed that the first five N-terminal amino acids of the recombinant protein from both yeasts were NH_2 -EPAVY, which corresponds the N-terminal sequence of mature human CRT protein after signal cleavage. Taken together, these results indicated that native ER signal sequence of human CRT protein is recognized and correctly processed in yeast cells, and this allows translocation of recombinant protein into the ER following unexpected secretion outside the yeast cell. Also, the results proved that secreted human calreticulin purified from yeast cells does not carry any modification.

Partial digestion of calreticulin with trypsin (according to Corbett et al., 2000, *Eur J Biochem.* 268:2558-65; Højrup et al., 2001, *J Biol Chem.* 275:27177-85) was used to show correct folding and Ca^{2+} binding of yeast-expressed hCRT. hCRT was diluted to 1 mg/ml concentration in storage buffer containing 3 mM CaCl_2 . Digestion was performed in 50 μl volume by adding 1 μl of 0.5 mg/ml trypsin (ratio of hCRT:trypsin was 100:1 (w/w)). In a control tube, calcium was removed by adding EDTA to 5 mM concentration. Controls without trypsin and EDTA were also used. The reaction was stopped after two time points, 10 min. and 60 min., by adding 1 mM PMSF. The samples were boiled, loaded onto the gels, and SDS-PAGE was performed. The results are shown in FIG. 13: Trypsin digestion of recombinant human calreticulin purified from *P. pastoris* culture medium. U— Untreated sample with 5 μg of hCRT, incubated at 37 $^\circ$ C. without calcium and EDTA; For other samples reaction was performed at 37 $^\circ$ C. in storage buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 3 mM CaCl_2). In the control tubes calcium was removed by addition of 5 mM EDTA. M—protein molecular weight marker (“Page Ruler Unstained protein ladder”, Fermentas, #26614). Analysis of *S. cerevisiae*-derived CRT is not shown, but the same results were obtained.

Digestion with trypsin revealed two CRT bands resistant to protease digestion—about 50 kDa and about 23 kDa, respectively. Partial digestion of human placental CRT with trypsin to ~ 50 kDa fragment was reported earlier by Højrup et al., 2001. Moreover, they reported that addition of Ca^{2+} increases proteolysis rate of native CRT. We also observed this effect (FIG. 13; compare hCRT samples with and without calcium after trypsin digestion). However, addition of calcium resulted in the resistance of ~ 23 kDa fragment of yeast-expressed hCRT to trypsin digestion (FIG. 13). It is in accordance with the data reported for rabbit CRT expressed

in *P. pastoris*, as after calcium binding ~27 kDa similar CRT band was also resistant to protease digestion (Corbett et al., 2000). Limited proteolysis with trypsin is regarded as the best test for the quality of recombinant calreticulin and is used for analysis of commercial CRT products (Abcam,

undergo thorough protein quality control throughout the yeast secretion pathway, which allows secretion of correctly folded proteins only; meanwhile *E. coli*-synthesized analog is purified by capturing all expressed hCRT molecules independently of their folding state.

TABLE 2

Cellular proliferation of human fibroblasts induced by hCRT expressed in bacteria, <i>S. cerevisiae</i> and <i>P. pastoris</i> , respectively.									
		Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Average	Error
		Fold Change	Fold Change	Fold Change	Fold Change	Fold Change	Fold Change		
Human CRT (Bacteria)	ng/mL								
	0	1	1	1	1	1	1	1	0.016294195
	0.1	1.133514986	1.259426848	1.103481625	0.989879823	1.158415842	1.325415677	1.161689133	0.024683693
	1	1.253405995	1.274509804	1.077369439	1.12397217	1.121287129	1.375296912	1.204306908	0.03343521
	10	1.389645777	1.273001508	1.138297872	1.064516129	1.193069307	1.472684086	1.255202446	0.021668366
	100	1.234332425	1.021116139	1.151837524	1.144212524	1.08539604	1.344418052	1.163552117	0.020869458
Human CRT (<i>S. cerevisiae</i>)	10% FBS	1.667574932	1.684766214	1.586073501	1.442125237	1.522277228	1.714964371	1.60296358	0.013341821
	0.1	1.495912807	1.455505279	1.147001934	1.154965212	1.274752475	1.356294537	1.314072041	0.011533147
	1	1.659400545	1.395173454	1.261121857	1.18342821	1.103960396	1.448931116	1.342002596	0.030760373
	10	1.613079019	1.375565611	1.211798839	1.275142315	1.245049505	1.51543943	1.37267912	0.023089937
	100	1.286103542	1.027149321	1.131528046	1.212523719	1.183168317	1.410926366	1.208566552	0.015389875
	Human CRT (<i>P. pastoris</i>)	0.1	1.008174387	1.562594268	1.091876209	1.201771031	1.056930693	1.368171021	1.214919602
1		1.177111717	1.358974359	1.282398453	1.216951297	1.128712871	1.337292162	1.250240143	0.022952194
10		1.29972752	1.524886878	1.235976789	1.187223276	1.180693069	1.622327791	1.341805887	0.019054
100		1.525885559	1.075414781	1.132495164	1.156862745	1.091584158	1.358669834	1.223485374	0.013052392

Note:

graphical view of the results is shown in FIG. 14.

ab15729, see “Properties”). Therefore, limited digestion with trypsin suggested that yeast-expressed human CRT is correctly folded Ca²⁺ binding protein and has similar properties to native human placental CRT (Højrup et al., 2001) and recombinant rabbit CRT expressed in *P. pastoris* (Corbett et al., 2000).

After demonstration of correct folding and calcium-binding properties of yeast-expressed hCRT, we have assessed biological activity of both *P. pastoris*- and *S. cerevisiae*-expressed human recombinant protein products. It was previously shown that recombinant human calreticulin expressed in bacteria *E. coli* improves wound healing in both murine and porcine animal models through multiple biological effects (Gold et al., 2006; Nanney et al., 2008; Gold et al., 2010; Greives et al., 2012). Therefore, it was especially important to compare *E. coli*- and yeast-expressed hCRTs in a parallel test of biological activity that directly relates to reported wound healing effects. For cellular proliferation and migration assays here we used the same human CRT from bacteria that was earlier shown to improve wound healing in vivo by causing a dose-dependent increase in epithelial migration and granulation tissue formation in both murine and porcine normal and impaired animal models of skin injury (Gold et al., 2006; Greives et al., 2012). Yeast-expressed hCRT proteins were tested in parallel with recombinant hCRT from bacteria (from M. Michalak, University of Alberta, Edmonton Alberta, Canada). In vitro assay for cellular proliferation was performed according to Nanney et al., 2008; and Greives et al., 2012. Briefly, human fibroblasts were seeded at 1,000 cells per well in 96 well plates and grown to 50% confluency. After 24 hours starvation in 0.5% FBS media, treatment was applied in 0.5% media for 24 hours. Cells were then incubated in MTS solution for 1 hour and absorbance was measured at 490 nm. Both *S. cerevisiae*- and *P. pastoris*-expressed human calreticulin showed significantly higher induction of human fibroblast proliferation compared to the same protein purified from bacteria *E. coli* (Table 2 and FIG. 14). This may be explained by the fact that yeast-secreted hCRT must

Another biological hCRT activity test was wound healing scratch plate assay (Nanney et al., 2008; Greives et al., 2012). Human fibroblasts were seeded at 10,000 cells per well in 24 well plates and grown to 80% confluency. After 24 hour starvation in 0.5% FBS media, a scratch was made in the middle of the well with a 200 µl pipet tip. Treatment was applied in 0.5% FBS media. Pictures were taken at 0 hours and 6 hours. Results showed that the average for *E. coli*-expressed hCRT induction of human fibroblast migration was slightly higher than for yeast-derived analogues, but within the range of error (Table 3 and FIG. 15). Both bacteria- and yeast-expressed hCRT showed significantly higher induction of cellular migration, than negative (0.5% FBS—Fetal Bovine Serum) and positive (10% FBS) controls, respectively. It may be concluded that yeast-expressed recombinant hCRT is biologically active and induces migration of human fibroblasts at the similar extent as hCRT analog from bacteria.

TABLE 3

Cellular migration of human fibroblasts induced by hCRT expressed in bacteria, <i>S. cerevisiae</i> and <i>P. pastoris</i> , respectively.					
Treatment	ng/mL	Percent migration Trial 1	Percent migration Trial 2	Average	Error
0.5% FBS		5.378067	5.60535	5.491708	1.144876
10% FBS		7.41555	6.80745	7.1115	0.733128
Human CRT (Bacteria)	1	9.2857	8.4337	8.8597	0.710761
	10	8.03795	10.625	9.331475	1.203801
	100	6.62775	8.590067	7.608908	0.421805
Human CRT (<i>S. cerevisiae</i>)	1	8.203467	5.88745	7.045458	0.729564
	10	6.90875	8.869667	7.889208	0.832518
	100	9.46755	7.8812	8.674375	0.696941
Human CRT (<i>P. pastoris</i>)	1	8.24245	8.02525	8.13385	0.988465
	10	8.79435	8.6602	8.727275	0.342841
	100	8.0294	6.91955	7.474475	0.800834

Note:

graphical view of the results is shown in FIG. 15.

Taken together, here we demonstrate that the native signal of human calreticulin is correctly cleaved and drives secretion in yeast cells. Sequence of yeast-secreted human calreticulin fully corresponds to mature protein from human cells, and protein is free of yeast-derived modifications. Therefore, yeast cells are excellent host for production of large amounts of correctly folded native recombinant human calreticulin.

Example 3

Native Signal Peptide of Human ERp57 Disulfide Isomerase Mediates Secretion of Active Native Recombinant ERp57 Protein in Yeast *Saccharomyces cerevisiae* and *Pichia pastoris*

Human ERp57 protein is mainly glycoprotein specific disulfide isomerase, which facilitates folding of glycoprotein precursors in the ER in the concert with ER lectin chaperones calreticulin and calnexin.

A growing amount of data also associates ERp57 protein with many different functions in other subcellular locations outside the ER. Those functions are often not well understood and require further studies and deeper analysis. Analysis of protein functions generally requires relatively large amounts of biologically active protein, which as much as possible resembles its native state.

Yeast *Saccharomyces cerevisiae* were introduced as a perfect host for production of human ERp57 protein. We found that endogenous signal peptide of human ERp57 protein was recognized and correctly processed in yeast cells, which subsequently leads to the secretion of the ERp57 protein. Secreted recombinant ERp57 protein possessed native amino acid sequence and was biologically active. Moreover, secretion allowed simple one-step purification of native recombinant human ERp57 protein, with yields up to 10 mg/L.

This example shows that native signal peptide of human ERp57 was correctly processed in *S. cerevisiae*, that native sequence human ERp57 was secreted in yeast *S. cerevisiae*, and that secreted native recombinant human ERp57 was biologically active.

Enzymes and kits for DNA manipulations were from ThermoScientific. Primers were from IDT.

For plasmids, strains, media, yeast transformation and cultivation, all DNA manipulations were performed according to standard procedures (Sambrook and Russell, 2001). Bacterial recombinants were screened in *Escherichia coli* DH5 α F' cells. The yeast *S. cerevisiae* strain AH22 MATa leu2 his4 was used for expression experiments. Transformation of *S. cerevisiae* cells was performed by conventional methods (Sambrook and Russell, 2001). The selection of transformants resistant to formaldehyde was carried out on YEPD (yeast extract 1%, peptone 2%, dextrose 2%) agar supplemented with 4 mM formaldehyde. *S. cerevisiae* transformants were grown in YEPD medium supplemented with 4 mM formaldehyde.

For protein expression and purification, yeast cells carrying the human PDIA3 gene were grown for 36 h in YEPD medium. Cells were separated from the medium by centrifugation at 2000 \times g for 10 min. Yeast growth medium was further prefiltered through qualitative filter paper (VWR, cat. no. 516-0812) with subsequent microfiltration through 1.6 μ M (SartoriusStedim Biotech, cat. no. FT-3-1101-047), 0.45 μ M (SartoriusStedim Biotech, cat. no. 15406-47) and 0.2 μ M (SartoriusStedim Biotech, cat. no. 15407-47-MIN) filters. After microfiltration, proteins from the medium were

concentrated and transferred into the binding buffer (20 mM Tris-HCl, pH 8.0) through tangential ultrafiltration using cassettes with 50 kDa cut-off membranes (SartoriusStedim Biotech, cat. no. VF20P3). Proteins were incubated for 30 min with heparin sepharose (GE Healthcare, cat. no. 17-0998-01) in batch format. Unbound proteins were removed while bound proteins were eluted with a step NaCl gradient (150 mM—250 mM—350 mM). Elution fractions were analyzed by SDS-PAGE. All showed more than 90% pure human ERp57 protein. All fractions were pooled and dialysed against 20 mM Tris-HCl, pH 8.0 NaCl 100 mM buffer.

Insulin turbidity assay was performed as described (Hirano et al., 1995; Frickel et al., 2004). The assay mixture was prepared in a cuvette by addition of 50 μ l insulin (Sigma-Aldrich, cat. no. 12643) (1 mg/ml in 100 mM potassium acetate pH 7.5, 2 mM EDTA) plus tested protein and water for a final volume of 60 μ l. The reaction was started by pipetting 2 μ l dithiothreitol (10 mM) in a cuvette. The cuvette was then thoroughly mixed and placed in the spectrophotometer (Tecan's Infinite M200). Measurements were performed at 650 nm using 60-s recordings. Assays lasting up to 60 min were not mixed further. The onset of aggregation was defined as the time where OD650 had reached the value of 0.025. The enzyme concentration at which this occurred was plotted against the onset of aggregation in order to obtain a concentration-dependent activity curve for the reductase activity of each oxidoreductase. *E. coli* thioredoxin was purchased from Sigma-Aldrich (cat. no. T0910) and recombinant human ERp57, produced in *E. coli*, was purchased from Nordic BioSite (cat. no. PAT-80438-1) were used as controls.

N terminus sequencing of yeast secreted human ERp57 protein by Edman degradation was performed by AltaBioscience. The molecular mass of protein was measured by electrospray mass spectrometry using Agilent Q-TOF 6520 mass spectrometer. Protein concentrations were determined by Roti-Nanoquant Protein-assay (Carl Roth GmbH., cat. no. K880).

Densitometric analysis of SDS-PAGE gels, scanned with ImageScanner III (GE Healthcare) were performed with ImageQuant TL (GE Healthcare) software using default settings.

Precipitation of proteins from yeast growth medium for SDS-PAGE analysis was performed based on a defined methanol-chloroform-water mixture as described (Wessel and Flügge, 1984).

For construction of human ERp57 yeast expression vector, ERp57 coding gene (PDIA3, Acc. no. U42068) was cloned under constitutive yeast PGK1 promoter in pFDC vector, yielding pFDC-hERp57 plasmid as it was described previously (Čiplys et al., 2011). Briefly, human PDIA3 gene was cloned from human adult liver cDNA library (Clontech) using primers that generate XbaI restriction sites on both ends of the gene, allowing restriction cloning into the XbaI site of pFDC vector between yeast PGK1 promoter and terminator. Yeast expression vector pFDC-hERp57 was transformed into the *S. cerevisiae* strain AH22. Yeast cells harboring human PDIA3 gene were grown in YEPD medium and secreted native recombinant human ERp57 protein was purified to 90% purity as described above. The purification procedure is illustrated in FIG. 16: A—crude yeast growth medium (20 \times concentrated), B—yeast growth medium after microfiltration (20 \times concentrated), C—20 \times concentrated proteins from yeast growth medium in binding buffer after tangential ultrafiltration; D—purified yeast-derived recombinant human ERp57 protein (2 μ g), M—un-

stained protein ladder (ThermoScientific, cat. No. 26614). According to data obtained from densitometric analysis of SDS-PAGE gels, secreted human ERp57 protein constitutes for approx. 30% of all yeast secreted protein (FIG. 16 lane A), subsequent microfiltration increases its purity to approx. 50% (FIG. 16 lane B) and one-step affinity chromatography using heparin Sepharose is enough to reach over 90% purity (FIG. 16 lane D). Yields obtained were approx. 9 mg from 1 L culture medium with purification efficiency reaching up to 90%. In summary, secretion of human ERp57 into the yeast growth medium allows simple and cost-effective purification of native recombinant protein.

N-terminal sequencing by Edman degradation was performed for identification and characterization of purified secreted protein. The first five N-terminal amino acids of the recombinant protein were NH₂—SDVLE, which corresponds the N-terminal sequence of mature human ERp57 protein after signal cleavage (Charnock-Jones et al., 1996). These results indicate that native ER signal sequence of human ERp57 protein is recognized and correctly processed in yeast cells, and this allows translocation of recombinant protein into the ER following unexpected secretion outside the yeast cell. *S. cerevisiae* alpha-mating factor signal sequence is usually employed for secretion of recombinant proteins in yeast, since native secretion signals are rarely effective (Sleep et al., 1990; Ferrarese et al., 1998; Guo and Ma, 2008), even though usage of native secretion signals offers several advantages. First of all, it simplifies the cloning of the gene, and, most importantly, it allows secretion of recombinant protein without any additional amino acids, as the disclosed data illustrate. In contrast, some additional amino acids are usually introduced into recombinant product when using non-native signal sequences (Andrin et al., 2000).

Mass spectrometry results of yeast-derived purified human ERp57 protein showed the mass of 54265.55 Daltons, which exactly corresponds to theoretically predicted mass of mature human ERp57 (25-505 aa) (FIG. 17). It indicates two things: (a) recombinant human ERp57 protein is exactly the same polypeptide as mature human ERp57 (including predicted ER retention signal QEDL on the C terminus of the protein) and (b) it has no yeast-derived modifications—a very important characteristic for recombinant proteins. Moreover, mass spectroscopy analysis revealed that the protein was highly pure (FIG. 17). In addition, we performed tryptic peptide mass fingerprint analysis of *S. cerevisiae*-purified recombinant secreted ERp57 and have identified N-terminal peptide SDVLELTDDNFESR (FIG. 18, indicated in bold), which corresponds to the N-terminus of mature human ERp57 protein, identified in a database search as PDIA3_Human (Acc. No. P30101). Together with N-terminal sequencing and ESI-MS data this once more demonstrates correct processing of the secreted recombinant human ERp57 protein product.

The presence of intact QEDL sequence in recombinant human ERp57, which usually serves as ER retention signal in human cells, raises question about reasons of secretion of human protein by yeast cells and about retention of proteins in the ER in general. In some cases ERp57 was found on the surface of the human cells, and this suggests several important functions for the protein (Turano et al., 2011). Secretion of human ERp57 protein by *S. cerevisiae* cells could be explained by yeast preference for the HDEL rather than QDEL signal for the retrieval of ER-residing proteins (Dean and Pelham, 1990), but it is not the reason in this case, because replacement of QEDL with the HDEL sequence did not suppress the secretion of ERp57 (our unpublished data).

Also, overload of the yeast ER retrieval machinery can be omitted as the reason for secretion of human ERp57, because overexpression of yeast KAR2 protein with native HDEL ER retrieval sequence using the same pFDC vector did not lead to the secretion of this protein (our unpublished data). Moreover, human PDI, which is homologue of human ERp57 and yeast PDI proteins and contains KDEL ER retrieval sequence, was also expressed using the same pFDC vector, and in this case secretion of recombinant protein was not observed (our unpublished data). These experiments indicate that retention of ER luminal proteins is complicated and still unsolved mechanism, which does not strictly depend on HDEL/KDEL retrieval mechanism. Our finding, secretion of human ERp57 by yeast cells, could serve as a convenient model for studying this phenomenon.

Activity of yeast derived recombinant human ERp57 protein was measured by an insulin turbidity test that is often used for characterization of protein disulfide isomerases (Hirano et al., 1995; Antoniou et al., 2000; Frickel et al., 2004; Celli and Jaiswal, 2003). Recombinant ERp57 exhibited thiol-dependent reductase activity which catalyzes the reduction of insulin disulfides by dithiothreitol (FIG. 19). Reductase activity of the protein was compared to *E. coli* thioredoxin and commercially available *E. coli*-derived recombinant human ERp57. Thiol-dependent catalytic activity of yeast-derived recombinant human ERp57 protein assayed using the insulin precipitation method is shown in FIG. 19: Various concentrations of *E. coli* thioredoxin (◆), purified *S. cerevisiae* secreted human recombinant ERp57 (■) and human recombinant ERp57 purified from *E. coli* (▲) were tested for their ability to catalyze the reduction of 130 mM insulin by 0.33 mM DTT. The onset of aggregation was defined as the time when the optical density at 650 nm had reached a value of 0.025 and was plotted against the concentration of catalyst used. Data are the average of three independent experiments. Error bars were too small to be visible. The activity of recombinant *E. coli*-derived human ERp57 at higher concentrations was not measured due to the absence of large amount of the protein. As shown in FIG. 19, both recombinant human ERp57 proteins catalyzed the reduction of insulin in slower rate than thioredoxin, in agreement with the results of the previous studies (Hirano et al., 1995; Frickel et al., 2004). Nevertheless, activity of yeast-secreted human ERp57 was slightly but reliably higher than that of *E. coli*-derived protein (note—higher activity of the proteins is represented by the lower position on Y axis in FIG. 19 diagram, as shorter time for insulin precipitation indicates faster catalization of the reaction). This may be explained by the fact that yeast-secreted human ERp57 must undergo thorough protein quality control throughout the yeast secretion pathway, which allows secretion of correctly folded proteins only, meanwhile *E. coli*-synthesized ERp57 is purified by capturing all histidine tag-containing proteins independently of their folding state. In summary, our method for production of native recombinant human ERp57 yields active protein, thus enabling its application in various studies.

Together with *S. cerevisiae* we have also performed human ERp57 expression experiments in yeast *P. pastoris* system. The whole cDNA of PDIA3 gene was cloned into pPIC3.5K vector under control of AOX1 promoter, similarly as in the cases of expression of human genes encoding BiP and calreticulin proteins. However, in the case of ERp57 the expression under standard conditions recommended by manufacturer (Invitrogen) was less successful as ERp57 secretion level was much lower than that of secretion of this protein in *S. cerevisiae* (not shown). Then we used opti-

mized *P. pastoris* culturing conditions at 20° C. in growing medium containing 1% YNB, 2% peptone, 1% yeast extract, buffered by 100 mM potassium phosphate (pH 6.0) and including 1% glycerol or 1% methanol for generation of yeast biomass or induction of PDIA3 gene expression, respectively. Under these optimised conditions we achieved high-level secretion of ERp57 into the culture medium. A clear human ERp57 protein band was visible in SDS-PAGE after loading of unconcentrated culture medium sample (FIG. 3, panel C lane rERp57). Then the protein was purified from *P. pastoris* culture medium using exactly the same method as for *S. cerevisiae*-expressed ERp57. Purification procedure has yielded ~30 mg of purified human protein from 1 liter of *P. pastoris* culture medium. SDS-PAGE analysis of *P. pastoris*-derived human ERp57 protein is shown in FIG. 20. 5 µg of purified protein was loaded onto SDS-PAA gel lane. N-terminal sequencing by Edman degradation displayed the same N-terminal amino acid sequence NH₂—SDVLE for *P. pastoris*-expressed human ERp57. As it is noted above, the same result was obtained for analogue protein expressed in *S. cerevisiae*. Therefore, both yeasts correctly processed and secreted large amounts of native recombinant human ERp57 protein.

This Example provides a simple method for production of native recombinant human ERp57 protein. The exemplified system, using both yeast *S. cerevisiae* and *P. pastoris* cells, allowed production of human thiol-disulfide oxidoreductase ERp57 in eukaryotic endoplasmic reticulum, where the environment is well suited for maturation of such proteins. The disclosed method demonstrated that the native signal of human ERp57 protein was correctly cleaved and drove its secretion outside the yeast cells. Amino acid sequence of secreted native recombinant human ERp57 protein fully corresponded to mature protein from human cells with no yeast derived modifications. Secretion of human ERp57 protein into the yeast medium not only allowed effective simple and cost-effective one-step purification of the protein, but also ensured its higher activity compared to *E. coli* produced ERp57 protein. Yeast was the perfect host for production of human ERp57 protein and also could serve as a convenient model for studying retention of ER luminal proteins in the ER.

The embodiments shown and described in the specification are only specific embodiments of inventors who are skilled in the art and are not limiting in any way. Therefore, various changes, modifications, or alterations to those embodiments may be made without departing from the spirit of the invention in the scope of the following claims. The references cited are expressly incorporated by reference herein in their entirety.

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-continued

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His Ile Pro Asp Pro Asp Ala Lys Lys Pro Glu Asp Trp Asp Glu Glu
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Met Asp Gly Glu Trp Glu Pro Pro Val Ile Gln Asn Pro Glu Tyr Lys
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Gly Glu Trp Lys Pro Arg Gln Ile Asp Asn Pro Asp Tyr Lys Gly Thr
 260 265 270

Trp Ile His Pro Glu Ile Asp Asn Pro Glu Tyr Ser Pro Asp Pro Ser
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Ile Tyr Ala Tyr Asp Asn Phe Gly Val Leu Gly Leu Asp Leu Trp Gln
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Val Lys Ser Gly Thr Ile Phe Asp Asn Phe Leu Ile Thr Asn Asp Glu
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Ala Tyr Ala Glu Glu Phe Gly Asn Glu Thr Trp Gly Val Thr Lys Ala
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Val Ser Gly Tyr Pro Thr Leu Lys Ile Phe Arg Asp Gly Glu Glu Ala
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 425 430 435 440
 Lys Lys Tyr Glu Gly Gly Arg Glu Leu Ser Asp Phe Ile Ser Tyr Leu
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What is claimed is:

1. A method for producing a mature human endoplasmic reticulum chaperone protein, wherein the mature human ER chaperone protein is a human ER luminal protein, the method comprising:

- (a) providing a yeast cell transformed with a full-length human chaperone cDNA encoding a polypeptide comprising a native signal sequence and the mature human ER chaperone protein;
- (b) culturing the yeast cell in a culture medium and allowing the yeast cell to express the polypeptide;
- (c) allowing the yeast cell to recognize and correctly process the native signal sequence of the polypeptide to thereby provide the mature human ER chaperone protein;
- (d) allowing the yeast cell to secrete the mature human ER chaperone protein outside the yeast cell and into the culture medium; and
- (e) extracting the mature human ER chaperone protein from the culture medium.

2. The method of claim 1, wherein the yeast is *Saccharomyces cerevisiae*.

3. The method of claim 1, wherein the yeast is *Pichia pastoris*.

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4. The method of claim 1, wherein the human endoplasmic reticulum chaperone protein is selected from the group consisting of BiP/GRP78, calreticulin, and ERp57.

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5. The method of claim 4, wherein the human endoplasmic reticulum chaperone protein is BiP/GRP78 protein, and the yeast is *Saccharomyces cerevisiae*.

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6. The method of claim 4, wherein the human endoplasmic reticulum chaperone protein is BiP/GRP78 protein and the yeast is *Pichia pastoris*.

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7. The method of claim 4, wherein the human endoplasmic reticulum protein is calreticulin protein, and the yeast is *Saccharomyces cerevisiae*.

8. The method of claim 4, wherein the human endoplasmic reticulum protein is calreticulin protein and the yeast is *Pichia pastoris*.

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9. The method of claim 4, wherein the human endoplasmic reticulum protein is ERp57 protein and the yeast is *Saccharomyces cerevisiae*.

10. The method of claim 4 wherein the human endoplasmic reticulum protein is ERp57 protein and the yeast is *Pichia pastoris*.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 9,796,971 B2
APPLICATION NO. : 14/594485
DATED : October 24, 2017
INVENTOR(S) : Ciplys et al.

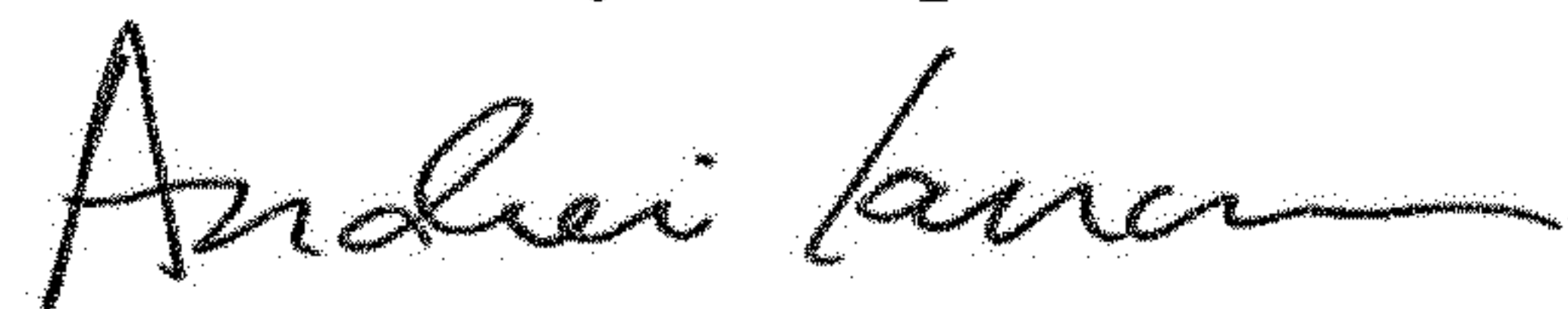
Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title Page

Item (72) should be amended to remove inventors Marek MICHALAK and Leslie Ina GOLD.

Signed and Sealed this
Eleventh Day of September, 2018



Andrei Iancu
Director of the United States Patent and Trademark Office